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THE STIMULATION OF ALDOSTERONE SECRETION BY ADRENOCORTICOTROPIC HORMONE (ACTH)*

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ABSTRACT

When the urinary excretion of aldosterone is compared to that of cortisol, the rise in the former due to administration of ACTH is quantitatively negligible, as appears from acute and chronic studies on 4 human subjects. However, the data presented suggest that ACTH stimulates the production of aldosterone as well as that of cortisol.

STUDIES on the urinary output of steroids indicate that adrenocorticotropic hormone (ACTH) is not the principal factor controlling the secretion of aldosterone by the adrenal cortex (1–5). The amount of aldosterone excreted by patients without pituitary function is only slightly de-

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creased at most (5–8). However, some rise occurs following administration of ACTH (9–12). Quantitative, adequate comparison between the response of cortisol and the response of aldosterone to ACTH stimulation is difficult, since the secretion of aldosterone is inferred from the amount excreted unchanged in the urine, whereas the secretion of cortisol is usually evaluated by the measurement of several of its metabolites. It seemed of interest to compare the simultaneous quantitative measurements of cortisol and aldosterone in urine, before and during stimulation with ACTH.

MATERIAL AND METHODS

The studies were carried out in the Metabolic Ward of the Peter Bent Brigham Hospital. A dose of 25 units of lyophilized ACTH was administered intravenously in 500 ml. of 5 per cent dextrose over eight hours on two consecutive days in 5 instances, and on four consecutive days in 1 instance. Patient R.A., a 42-year-old male suffering from a benign spinal cord tumor, underwent the test twice while he was receiving a normal sodium intake, and once when his sodium intake was reduced to 9 mEq. daily. Two other subjects who had an equally restricted sodium intake at the time of the test were Patient M.L., a 39-year-old female with intractable edema due to an idiopathic capillary permeability defect (13), and Patient A.P., a 35-year-old male with congestive cardiac failure due to rheumatic valvular disease. A fourth patient (F.B.), a 29-year-old female, presented the classic picture of Cushing's syndrome due to bilateral adrenal hyperplasia.

In addition, Patient R.A. received ACTH intramuscularly (20 units of ACTHAR-gel every twelve hours) for twenty-seven days, while receiving a diet containing 69 mEq. of sodium daily.

Urine collections (24-hour) were obtained before, during and after the administration of ACTH. The method used for the determination of aldosterone in urine was a modification (10) of that of Neher and Wettstein (14). Urinary cortisol was measured by a procedure described and discussed previously (5). No correction was attempted for losses due to the manipulations required for isolation of either steroid. 17-Hydroxycorticoids in urine were determined by the method of Reddy (15).

RESULTS

In Patient R.A., with normal adrenal function, the excretion of aldosterone in urine rose slightly during intravenous administration of ACTH (Fig. 1). The increase was more pronounced when the intake of sodium was minimal. The excretion of aldosterone was very low following administration of ACTH when the intake of sodium was normal. The rise in aldosterone excretion due to ACTH was much inferior to that of cortisol, which increased ten times, while the 17-hydroxycorticoids increased five times.

The same subject (R.A.) was maintained under the influence of ACTH for twenty-seven consecutive days. The difference between the degrees of response of aldosterone and of cortisol was accentuated during this prolonged stimulation of adrenocortical secretion. As seen in Table 1, the excretion of aldosterone rose to almost twice the control level during the sec-

ond half of the experimental period, contrasting with a 22-fold increase in the excretion of cortisol, and a 6-fold increase in the excretion of 17-hydroxycorticosteroids.

During the first ten days of the prolonged period of ACTH administration in Patient R.A. the sodium balance was frankly positive, as reflected by his gain in weight and average urinary excretion of 24 mEq. of sodium per twenty-four hours. During the latter two weeks, the sodium balance was markedly negative despite the elevated aldosterone values (Table 1).

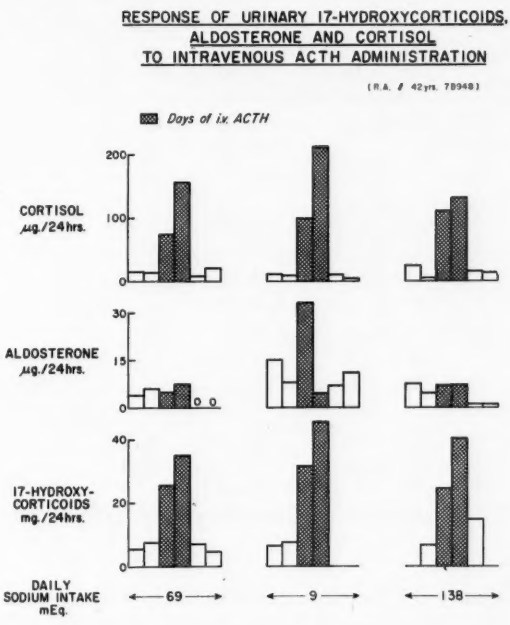

FIGURE 1

He excreted daily in the urine an average of 50 mEq. of sodium in excess of intake, and lost body weight accordingly.

As seen in Table 1, the response of adrenocortical secretion to ACTH decreased during the last days; this may possibly be explained by the development of resistance to intramuscular ACTH (16).

Measurements of urinary aldosterone and cortisol were carried out in 3 other subjects given ACTH intravenously. The findings were comparable to those obtained in Patient R.A. (Table 2). In Patients M.L. and A.P., the excretion of aldosterone rose during administration of ACTH, although not in a sustained fashion. After ACTH was stopped, there was a fall, as in

TABLE 1. EFFECT OF PROLONGED ADRENOCORTICAL STIMULATION WITH INTRAMUSCULAR ACTH ON URINARY STEROIDS (PATIENT R.A.)

Days	17-OH-corticoids (mg./24 hrs.)	Aldosterone (μg./24 hrs.)	Cortisol (μg./24 hrs.)
Control			
—	5.4	4	15
—	7.4	6	14
—	7.3	15	5
—	8.2	3	3
—	8.1	6	4
—	5.3	2	4
ACTH*			
13	74.5	16	516
14	43.5	10	101
15	40.5	21	367
18	67.4	5	97
19	51	7	247
20	56	13	70
21	44	10	106
25	15.4	5	39
26	18.5	10	32
27	15.3	11	52
Mean			
Control	6.9	6.1	7.5
ACTH	42.6	10,8	162.7

* 40 I.U. of ACTHAR-gel in two fractional intramuscular doses daily. Sodium intake, 69 mEq. daily.

Patient R.A. Urinary cortisol increased in both subjects to a level approximately fifteen times the control value. Patient F.B. deserves special mention because of the excessive adrenal response to ACTH—a typical feature of adrenal hyperplasia (17). This excessive response, manifested by a rise in urinary 17-hydroxycorticoids, was much more apparent in the values for urinary cortisol, which increased *ninety-fold* during ACTH stimulation! Urinary excretion of aldosterone quadrupled and remained above the control level after ACTH was discontinued, in contrast with the decrease observed in the other cases.

DISCUSSION

In this attempted comparison between the adrenal secretions of cortisol and aldosterone by means of reliance on their urinary excretion values, it is assumed that the metabolism and the renal handling of steroids are con-

TABLE 2. EFFECT OF ADRENOCORTICAL STIMULATION WITH INTRAVENOUS ACTH ON URINARY STEROIDS IN PATHOLOGIC CONDITIONS

Patient & Diagnosis	Days	17-OH-corticoids (mg./24 hrs.)	Aldosterone (μg./24 hrs.)	Cortisol (μg./24 hrs.)
M.L., female, 39 yrs.; massive edema; (low-sodium diet, 9 mEq./day)	1	—	16	16
	2	5.8	60	5
	3*	35.2	50	165
	4	54	113	209
	5	26.6	59	31
	6	11	20	11
F.B., female, 29 yrs.; adrenal hyperplasia (unrestricted diet)	1	12.4	6	6
	2	14.5	6	22
	3*	48.3	28	828
	4	65.8	20	1692
	5	14.2	17	111
	6	11.7	8	38
A.P., male, 35 yrs.; congestive heart failure; (low-sodium diet, 8 mEq./day)	1	—	19	7
	2	2.6	28	3
	3*	9.2	7	63
	4	13	37	70
	5	12.5	19	64
	6	12.5	12	84
	7	6.3	5	8
	8	4.7	8	15

* Patients M.L. and F.B. received 25 I.U. of ACTH in 500 ml. of 5 per cent dextrose in water, injected intravenously from 8 a.m. to 4 p.m., on two consecutive days (days 3 and 4). Patient A.P. received it on four consecutive days (days 3 to 6).

stant, whatever the circulating levels. For aldosterone, this has been shown to be true by Ayres *et al.* (18). However, not all of the plasma cortisol is firmly bound to proteins at high concentrations (19–21); the small free fraction presumably undergoes glomerular filtration (19). Thus the urinary excretion of cortisol is likely to rise excessively when ACTH is administered, as a result of the occurrence in the circulation of free cortisol.

Although the rise in the secretion of aldosterone implied from these studies appears quite small in comparison to that of other corticosteroids, it is probably part of the response of the adrenal cortex to ACTH, as sug-

gested by the following data: (a) The increase in aldosterone excretion due to ACTH can be roughly correlated with control levels; this was especially noticeable in Patient R.A., in whom the period of low-sodium diet (itself a stimulus to aldosterone secretion (1, 3, 4, 22)) resulted in elevated urinary levels already during the control days. (b) When Patient R.A. was exposed to prolonged ACTH stimulation, the urinary excretion of aldosterone remained increased despite the retention of sodium and water; this led to an important extracellular expansion that in itself would have produced a decrease in the adrenal secretion of this steroid (4), and is probably the reason for the post-ACTH low excretion of aldosterone in this patient when given a normal diet. (c) In Patient F.B., whose adrenal glands were excessively sensitive to ACTH, administration of ACTH stimulated briskly the secretion of both cortisol and aldosterone.

Additional evidence in favor of direct stimulation by ACTH of the adrenal secretion of aldosterone is found in the perfusion studies of Rosenfeld *et al.* with isolated beef adrenal glands (23), and in the incubation studies of Mialhe-Voloss and Baulieu using rat adrenal glands (24).

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INFLUENCE OF THE RENAL EXCRETION OF SODIUM CHLORIDE UPON THE RENAL EXCRETION OF MAGNESIUM AND OTHER IONS BY HUMAN SUBJECTS*

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ABSTRACT

Alterations in the rate of urinary excretion of magnesium in human subjects receiving a constant diet are consistently produced by imposing conditions resulting in pronounced changes in the rate of urinary excretion of sodium and chloride. Diminished urinary salt excretion, whether occurring as a result of reduced salt intake or of increased adrenocortical activity, is accompanied by diminished urinary magnesium excretion, and augmented urinary salt excretion produced by withdrawal of exogenous or endogenous adrenocortical hormones is accompanied by increased urinary magnesium excretion. Prevention of the usual effect of diminished adrenocortical activity upon urinary sodium chloride excretion also prevents its effect upon urinary magnesium excretion. Moreover, the effect of salt restriction upon urinary magnesium is equally great in normal subjects or in severely adrenal-deficient patients. Therefore adrenocortical hormones affect urinary magnesium excretion as a consequence of affecting urinary sodium chloride excretion, and no adrenocortical hormone mediates the changes produced in urinary magnesium excretion by varying the dietary salt intake. Other data suggest but do not prove that the observed changes in urinary magnesium excretion reflect primary alterations of renal magnesium clearance resulting from changes in net renal tubular reabsorption of filtered magnesium. It is not possible at present to define the relative importance of sodium versus chloride ions in relation to the observed effects of variation in the rate of urinary sodium chloride excretion. Limited but sig-

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nificant data show that when urinary sodium chloride excretion is altered, the rate of urinary excretion of calcium parallels that of sodium, chloride and magnesium.

NOT MUCH is known of the homeostatic mechanisms involved in the maintenance of the normal body content of magnesium and of factors capable of influencing them. The subject has recently been reviewed (1–4). Magnesium is absorbed from the gut and excreted in both urine and stool; but whereas increased oral intake of the ion leads to a compensatory increase in the rate of its elimination in both urine and stool, parenteral loading results in increased excretion in the urine only. Magnesium deprivation is followed by decreased excretion in both feces and urine. From these observations, it seems that renal magnesium excretion is responsive to the magnesium content of one or more compartments of body water, whereas absorption of magnesium in the gut is related rather to the magnesium content of the diet. Administration of acidifying salts (5, 6) and parathyroid activity appear to increase the excretion of both magnesium and calcium; other influences upon the rate of magnesium elimination have not been delineated with any precision.

The observations to be presented here are concerned with effects of an imposed variation in the rate of urinary excretion of sodium and chloride upon the rate of urinary excretion of magnesium and other cations. The data have previously been communicated in abstract form (7).

METHODS

The studies were conducted in the Metabolic Unit of the University Hospital. The effect of imposed conditions (*e.g.*, withdrawal of dietary sodium chloride) upon 24-hour urinary cation elimination and other variables was studied by comparing the data obtained during a three- or four-day "experimental period" with the data of a preceding (and sometimes also a succeeding) control period. The diet consumed per day was identical throughout each patient-study, save for the imposed change in mineral intake in certain subjects (Tables 1, 5, and 6). The only exceptions were Patient J.C., who was unable to take all her diet on the last day of the study and Patient M.H., who refused a small amount of food on the penultimate day. The initial control period was three days in some instances and four days in others.

Subjects included normal volunteers and patients with hypertensive disease or adrenal deficiency (see tables). In the cases of adrenalectomy, the operation had been performed for hypertensive disease. In the patients with spontaneous adrenal deficiency the diagnosis was based on the presence of pigmentation, episodes of acute adrenal insufficiency, and failure to respond to intravenous corticotropin with the conventional manifestations of adrenocortical activation.

Magnesium in urine was determined by the method of Heagy (8). Other analytical methods and the technique used for the balance study have been reported elsewhere (9, 10). The magnesium content of the diets was not determined, but throughout each patient-study the constancy of the daily intake of magnesium and other constituents was assured by preliminary pooling of the constituent foodstuffs from which the diets

TABLE 1. EFFECT OF REDUCTION OF DIETARY SODIUM CHLORIDE UPON URINARY EXCRETION OF SODIUM, CHLORIDE, POTASSIUM, MAGNESIUM AND CALCIUM

Degree of salt restriction*	Subjects				Intake per 24 hours (mean)				Urinary excretion per 24 hours (mean)									
					Control period		Salt restriction		Control					Salt restriction				
	Initials	Diagnosis*	Age & Sex (yrs.)	Surface area (sq. M.)	Na (mEq.)	Cl (mEq.)	Na (mEq.)	Cl (mEq.)	Na (mEq.)	Cl (mEq.)	Mg (mg.)	K (mEq.)	Ca (mg.)	Na (mEq.)	Cl (mEq.)	Mg (mg.)	K (mEq.)	Ca (mg.)
Moderate	M.P.	V	23 M	1.62	247	232	111	96	209	212	107	65	135	128	129	96	56	105
	L.K.	V	23 M	1.96	250	235	114	98	199	210	173	83	136	141	137	140	59	120
	B.L.	SA	18 F	1.42	262	258	108	104	236	239	102	70	—	124	104	71	68	—
	E.M. (I)	SA	37 M	2.01	248	234	111	97	232	233	130	72	—	145	144	102	69	—
	G.D.	AD	58 F	1.78	246	237	109	100	218	209	85	67	—	142	134	61	59	—
Drastic	M.K.	H	18 F	1.56	147	145	10	8	124	123	114	54	—	59	59	75	38	—
	F.N.	H	40 M	1.77	146	149	9	12	110	132	66	52	—	33	61	58	45	—
	E.M. (II)	SA	37 M	2.01	249	233	6	39	224	213	120	73	—	89	82	86	58	—

* See text.

** V = volunteer (normal); SA = subtotal adrenalectomy for hypertensive disease; AD = Addison's disease of spontaneous origin; H = hypertensive disease.

for the period were prepared. The significance of observed changes was evaluated with the aid of Fisher's *t* test (11).

RESULTS

Changes in urinary excretion of magnesium, sodium and chloride

Effect of dietary restriction of sodium chloride. The effect of salt withdrawal upon the excretion of cations in the urine was fairly uniform (Table 1). Urinary sodium and chloride excretion diminished markedly in all patients. Urinary magnesium excretion likewise diminished in all patients, the mean daily decrease ranging from 8 to 39 mg. and averaging 25.2 per cent of the mean control value. In 4 of 8 patients (L.K., B.L., G.D. and M.K.), the decrease was statistically significant ($P < .05$ to $P < .01$).

In 1 of the adrenal-deficient patients (G.D.) manifestations of severe adrenal insufficiency developed, necessitating termination of the study period early on the fourth day. In Patient E.M. (who was the subject of 2 studies) and in Patient B.L., the adrenal deficiency was subclinical; both had previously been subjected to subtotal adrenalectomy, did not require steroid replacement therapy, and did not show any ill effects of salt restriction in either of the studies shown in Table 1.

The amount of sodium chloride withdrawn was practically constant (about 137 mEq.) throughout, with the exception of study E.M.-II, in which a larger amount was withdrawn. However, only in the last 3 patients listed in Table 1 was salt restriction drastic, in the sense that the value to which the sodium and chloride intake was reduced during the individual study periods approached total salt deprivation.

Effect of corticotropin. Table 2 shows the effects of administration of corticotropin daily for six days to a patient with unimpaired adrenocortical reserve. In addition to the expected effect of corticotropin on the urinary

TABLE 2. EFFECT OF CORTICOTROPIN (40 UNITS DAILY GIVEN INTRAVENOUSLY IN EIGHT HOURS) ON URINARY EXCRETION OF SODIUM, CHLORIDE AND MAGNESIUM IN A PATIENT* WITH PEMPHIGUS

Period	Intake per 24 hrs. (mean)		Urinary excretion per 24 hrs. (mean)			
	Na (mEq.)	Cl (mEq.)	Na (mEq.)	Cl (mEq.)	Mg (mg.)	K (mEq.)
Control I (no hormone)	168	219	173	200	89	68
Corticotropin	172	222	27	84	68	79
Control II (no hormone)	163	216	273	281	104	45

* D.M., 46-year-old male; surface area 1.69 sq. M.

TABLE 3. EFFECT OF HORMONE WITHDRAWAL AND RESTITUTION UPON URINARY EXCRETION OF SODIUM, CHLORIDE, POTASSIUM, CALCIUM AND MAGNESIUM IN A TOTALLY ADRENALECTOMIZED PATIENT*

Period & Duration	Daily dosage of hormone**		Urinary excretion per 24 hrs. (mean)				
	DCA† (mg.)	E‡ (mg.)	Na (mEq.)	Cl (mEq.)	Mg (mg.)	K (mEq.)	Ca (mg.)
Control I (hormone; 4 days)	0	75	174	175	81	48	78
Withdrawal (no hormone; 3 days)	0	0	271	269	98	36	133
Control II (hormone; 4 days)	5§	50§	104	124	80	76	84

* B.J., 43-year-old male; surface area 1.94 sq. M.

** Sodium intake 224 mEq., chloride 206 mEq. per 24 hours, throughout.

† Desoxycorticosterone acetate in oil, intramuscularly.

‡ Cortisone acetate, orally.

§ Administered on the last three days of this period. On the first day of the period patient received 10 mg. of DCA (single intramuscular injection) and 175 mg. of cortisone in fractional oral doses.

excretion of sodium and chloride, there was a mean decrease of 21 mg. (30 per cent) per day in the urinary excretion of magnesium during the period of hormone administration, followed by a mean daily increase (comparing the second control period with the study period) of 36 mg. (53 per cent). These changes in the rate of excretion of magnesium were statistically significant.

Effects of cortisone (and withdrawal) and of hydrocortisone in adrenal-deficient patients. Table 3 shows the effects upon urinary electrolytes of withdrawal of cortisone therapy from a totally adrenalectomized male patient, and the effects of restitution of such therapy. During the period of hormone withdrawal, the urinary excretion of sodium, chloride and magnesium increased; the reverse occurred during the second control period when adrenal hormones were again administered. The respective mean changes in 24-hour magnesium excretion were +17 mg. (+21 per cent) and −18 mg. (−18 per cent). Values for urinary magnesium excretion on the second and third days of the withdrawal period were beyond the fiducial limits for control period I ($P = .001$), control period II ($P = .01$), and for the pooled control periods ($P = .001$).

Table 4 shows the effects of an infusion of hydrocortisone administered intravenously over a period of eight hours in an adrenal-deficient patient already receiving replacement therapy. Urinary sodium, chloride and magnesium excretion diminished on the day of hydrocortisone infusion as compared with the control period before and after.

TABLE 4. EFFECTS OF 8-HOUR CONTINUOUS INTRAVENOUS INFUSION OF 100 MG. OF HYDROCORTISONE UPON URINARY EXCRETION OF SODIUM, CHLORIDE, MAGNESIUM AND POTASSIUM IN AN ADRENAL-DEFICIENT PATIENT* RECEIVING REPLACEMENT THERAPY**

	Control I		Hydrocortisone (100 mg., i.v.)	Control II		
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Na (mEq.)	172	161	63	169	236	184
Cl (mEq.)	148	161	86	156	237	175
Mg (mg.)	35	40	23	58	52	35
K (mEq.)	58	62	81	74	81	57

* J.V., 41-year-old male; surface area 1.85 sq. M.

** Buccal desoxycorticosterone (4 mg. daily) and oral cortisone acetate (12.5 mg. daily) throughout.

Effect of simultaneous withdrawal of sodium chloride and adrenocortical hormones in adrenal-deficient patients. In Table 5 are shown the effects of reduction of dietary sodium chloride and simultaneous withdrawal of replacement adrenal hormones in 5 adrenal-deficient patients. Acute adrenal insufficiency developed in all 5 patients during the course of the study period. Reduction of urinary magnesium excretion occurred during the with-

TABLE 5. EFFECTS OF WITHDRAWAL OF SALT AND HORMONES UPON URINARY EXCRETION OF SODIUM, CHLORIDE, MAGNESIUM AND POTASSIUM IN 5 ADRENAL-DEFICIENT PATIENTS

Patients				Periods*	Intake per 24 hrs.				Urinary excretion per 24 hrs. (mean)			
Initials	Diagnosis†	Age & Sex (yrs.)	Surface area (sq. M.)		DCA‡ (mg.)	E§ (mg.)	Na (mEq.)	Cl (mEq.)	Na (mEq.)	Cl (mEq.)	Mg (mg.)	K (mEq.)
G.H.	AD	49 F	1.64	Control	5	12.5	215	213	255	251	40	57
				Withdrawal	0	0	110	109	177	167	36	51
S.W.	SA	47 M	1.98	Control	2	25	287	284	259	245	160	55
				Withdrawal	0	0	108	105	187	165	98	49
T.S.	SA	37 M	2.04	Control	4	25	253	241	297	305	118	60
				Withdrawal	0	0	115	103	160	157	75	51
M.H.	SA	31 F	1.65	Control	0	37.5	249	234	195	204	77	68
				Withdrawal	0	0	107	96	156	165	59	54
J.C.	SA	41 F	1.47	Control	2	37.5	161	158	179	159	39	56
				Withdrawal	0	0	98	96	133	123	45	57

* Control period in G.H. was of 4 days' duration; all other periods were of 3 days' duration.

† AD = Addison's disease of spontaneous origin; SA = subtotal adrenalectomy for hypertensive disease.

‡ Desoxycorticosterone acetate, buccal.

§ Cortisone acetate, oral.

drawal period in all patients except J.C.; the mean 24-hour decrease ranged from 4 to 62 mg. (10 to 37 per cent). Including the values for Patient J.C., there was an average decrease of 18.1 per cent in mean daily magnesium excretion during the withdrawal period. The observed reductions were statistically significant in 3 patients (S.W., T.S. and M.H.).

Actually the withdrawal period included a fourth day for Patients S.W., T.S. and J.C.; but because corticotropin was administered on this day, the values were not included in computing the mean values listed in

TABLE 6. EFFECT OF SUBSTITUTION OF EQUIVALENT QUANTITIES OF NH_4Cl AND OF $NaHCO_3$ RESPECTIVELY FOR NaCl IN THE DIET UPON THE URINARY EXCRETION OF SODIUM, CHLORIDE, MAGNESIUM AND CALCIUM IN 2 NORMAL VOLUNTEERS

Subject		Control period I	Substitution period	Control period II	Δ_1*	P	Δ_2**	P
N.M.†	Urinary 24-hr. excretion (mean values):	NaCl supplement (155 mEq./d.)	NH_4Cl supplement (152 mEq./d.)	NaCl supplement (155 mEq./d.)				
	Na (mEq.)	219.5	161	181	− 58.5		+20	
	Cl (mEq.)	224	245	238.5	+ 21		− 6.5	
	Mg (mg.)	117	145	116	+ 28	<.001	−29	<.01
	Ca (mg.)	254.5	366	302	+111.5	<.001	−64	<.001
L.K.‡	Urinary 24-hr. excretion (mean values):	NaCl supplement (155 mEq./d.)	$NaHCO_3$ supplement (155 mEq./d.)	NaCl supplement (155 (mEq./d.)				
	Na (mEq.)	234	284	229	+ 50		−55	
	Cl (mEq.)	233	141.5	220.5	− 91.5		+79	
	Mg (mg.)	175.5	148	163	− 27.5	<.01	+15	N.S.
	Ca (mg.)	221	134	228.5	− 87	<.01	+94.5	<.001

* Mean daily value for the experimental period minus mean daily value for control period I.
** Mean daily value for control period II minus mean daily value for the experimental period.
† Male, 23 years old; surface area 1.75 sq. M.
‡ See Table 1.

Table 5, even though there is little response to corticotropin in patients with this grade of adrenal deficiency (10). Inclusion of these fourth-day values would make the decrease of urinary magnesium more pronounced and more significant in Patients S.W. and T.S., and the increase less pronounced and less significant in Patient J.C.

Effect of substitution of ammonium chloride or sodium bicarbonate for sodium chloride in the diet. In Table 6 are shown the effects upon electrolyte excretion of the substitution of approximately chemically equivalent amounts of ammonium chloride or of sodium bicarbonate for sodium chloride in the diet of 2 normal volunteers. The substitution of ammonium chloride for sodium chloride resulted in an increased excretion of chloride and magnesium while sodium excretion decreased; the opposite changes were noted when sodium chloride replaced ammonium chloride. Substitu-

tion of sodium bicarbonate for sodium chloride resulted in decreased urinary excretion of chloride and magnesium and increased excretion of sodium; the reverse changes were seen when sodium chloride was substituted for the sodium bicarbonate.

Changes in serum magnesium concentration

In Figure 1 are shown serial determinations of serum magnesium concentration in 7 patients in whom acute adrenal insufficiency developed in the course of the studies. Generally, though not invariably, small but pro-

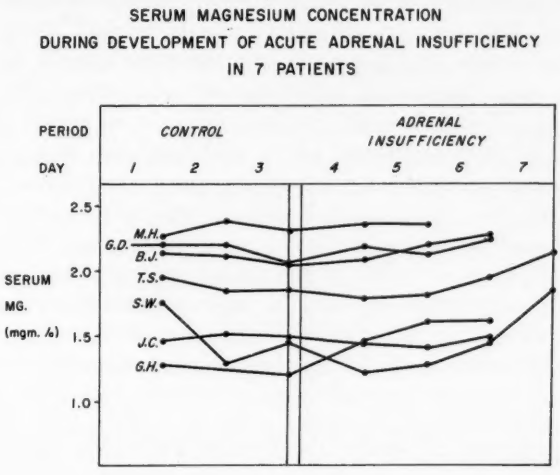

FIGURE 1

gressive increases of serum magnesium concentration accompanied adrenal insufficiency, especially when the period of hormone withdrawal lasted as long as four days. In none of the other subjects was any appreciable variation of serum magnesium concentration observed.

Changes in urinary excretion of potassium

Potassium excretion decreased in all subjects subjected to salt withdrawal alone (Table 1). The mean daily decrease ranged from 2 to 24 mEq. and averaged 15.7 per cent of the mean control value. The decrease was significant in 2 subjects (L.K. and G.D.). Simultaneous withdrawal of sodium chloride and replacement steroid therapy (Table 5) also led to a decrease in potassium excretion in 4 of 5 adrenal-deficient patients. The mean daily decrease ranged from 6 to 14 mEq.; the mean percentile change for all 5 patients was −10.4. In none of these periods was the decrease of urinary potassium statistically significant. Urinary potassium excretion

also fell when cortisone acetate was withdrawn from a totally adrenalectomized patient (Table 3) and rose during restitution of hormone therapy.

Infusion of corticotropin (Table 2) and of hydrocortisone (Table 4) led to the expected increase in potassium excretion.

Changes in urinary excretion of calcium

Urinary calcium excretion was measured in 2 normal volunteers subjected to salt restriction (Table 1), and in the totally adrenalectomized patient during withdrawal and restitution of adrenal steroid therapy (Table 3). Calcium excretion regularly paralleled sodium excretion in these studies. One of the volunteers showed a mean decrease of 30 mg. of calcium per twenty-four hours (22.2 per cent), and the other a mean decrease of 16 mg. (11.8 per cent); the former value represented a statistically significant change. In the adrenal-deficient patient, calcium excretion increased 71 per cent during the experimental period and decreased 36 per cent during the second control period when adrenal hormones were readministered. The increase during the experimental period was statistically significant whether compared with the data of the first control period ($P < .05$), the second control period ($P < .02$), or the pooled control period ($P < .01$).

In the study summarized in Table 6, calcium excretion paralleled chloride rather than sodium excretion when ammonium chloride or sodium bicarbonate was substituted for dietary sodium chloride in equivalent amounts.

Nitrogen balance, eosinophil counts, and urinary excretion of phosphorus and uric acid

The effect of restriction of dietary salt upon nitrogen balance was determined (not shown) in conjunction with the first 5 studies listed in Table 1. Changes were small in magnitude and there was no consistent change in direction during the period of restriction; neither were there any significant or consistent changes in circulating eosinophil counts and urinary excretion of phosphorus and uric acid (also not shown).

Inulin clearance and "24-hour endogenous creatinine clearances"

The "24-hour clearance of endogenous creatinine chromogen" was determined in the 2 volunteers subjected to salt restriction and in 4 of the patients in whom adrenal insufficiency developed; in 2 of the latter, inulin clearances were also measured on the last days of the first control period and of the withdrawal period (Table 7). The decrease of glomerular filtration rate which generally accompanies adrenal insufficiency was reflected in the measurements, which also illustrate the parallel movement of inulin clearance and "24-hour creatinine clearance" when these were both esti-

TABLE 7. CLEARANCE OF INULIN AND "24-HOUR CLEARANCE OF ENDOGENOUS CREATININE" (ML./MIN.)

			Control period I	Withdrawal period	Control period II
Subjects in whom adrenal insufficiency did not develop					
L.K. (Table 1)	Salt withdrawn	Mean C_{Cr}	86.2	104	
M.P. (Table 1)	Salt withdrawn	Mean C_{Cr}	88.6	86.2	
Patients in whom adrenal insufficiency developed					
B.J. (Table 3)	Hormone withdrawn	$C_{In}\pm SEM$	106.5±5.8	92.2±5.3	
		Mean C_{Cr}	82.8	81.6	84.7
M.H. (Table 5)	Hormone and salt withdrawn	$C_{In}\pm SEM$	61.4±5.3	47.8±7.2	
		Mean C_{Cr}	35.7	30.8	
T.S. (Table 5)	Hormone and salt withdrawn	Mean C_{Cr}	49.8	45.2	
G.D. (Table 5)	Salt withdrawn	Mean C_{Cr}	63.9	59.1	

mated (Patients B.J. and M.H.). The change in inulin clearance was considerably larger than the mean change in creatinine clearance in both instances. This is to be expected, since inulin clearance was measured only on the last day of the withdrawal period instead of representing the mean for the period. The mean proportional decrease in creatinine clearance during adrenal insufficiency was 7.9 per cent; in the 2 volunteers during salt restriction it was −2.6 and +6.2 per cent.

DISCUSSION

Table 8 has been constructed as an aid for the ensuing discussion; it is derived from data presented in detail in Tables 1–5, and presents in summary form the effects of the various imposed conditions upon the urinary excretion of magnesium and sodium chloride. The data are interpreted to mean that there is a link between the renal excretion of magnesium and that of sodium or chloride[1] such that large changes in the rate of excretion of the latter ions entail, as a consequence, parallel changes in the rate of excretion of magnesium. The linkage is in turn most readily visualized as a change in the renal tubular reabsorption of magnesium occurring in consequence of, and parallel to, a change in tubular reabsorption of sodium or chloride.

The rate of urinary excretion of magnesium as a function of the rate of urinary excretion of sodium or chloride

With one exception (J.C., Table 5), there was strict parallelism between the changes in urinary excretion of sodium chloride and magnesium produced by any one of the several imposed conditions (Table 8, columns 3 and 4).

[1] Or both: data do not permit a conclusion as to whether the observed effects are to be ascribed to a change in the rate of excretion of both ions together or of one or the other individually. Hereinafter this qualification will be assumed.

TABLE 8. SUMMARY OF EXPERIMENTAL CONDITIONS IMPOSED, TABLES 1 THROUGH 5, AND THEIR EFFECTS

Imposed conditions		Effects		Evidence	
Dietary NaCl	Adrenocortical activity	Urinary Na and Cl excretion	Urinary Mg excretion	Table	Period
1. ↓	(None imposed, but aldosterone secretion presumably increased when adrenal function intact. See text.)	↓	↓	1	Experimental
2. ↓	↓	↓	↓	5	Experimental
3. Unchanged	↓	↑	↑	2 4 3	Second control Second control Experimental
4. Unchanged	↑	↓	↓	2 4 3	Experimental Experimental Second control

In logic, such a parallelism can be interpreted in 4 ways: 1) It might represent a fortuitous conjunction of essentially independent effects of the several imposed conditions upon magnesium excretion on the one hand and sodium and chloride excretion on the other. 2) The imposed conditions might have affected some physiologic change which in turn affected the urinary excretion of magnesium and of sodium and chloride. 3) As previously suggested, the changes in urinary magnesium excretion might have been secondary to the changes in sodium and chloride excretion; or 4) the observed changes in urinary sodium and chloride excretion might be dependent upon the change in magnesium excretion.

This last explanation seems preposterous. It would imply that the large changes in urinary sodium chloride excretion observed when dietary sodium chloride was restricted were a consequence of the smaller changes in urinary magnesium excretion. This could hardly be the case; for when urinary magnesium excretion is manipulated by varying the magnesium load, the secondary parallel changes observed in the excretion of sodium and chloride are relatively small (6, 12–15).

The possibility must be considered that the several imposed conditions affect renal magnesium excretion independently of their effects upon renal sodium and chloride excretion, the parallelism observed being fortuitous

rather than the reflection of a physiologic liaison. It appears, however, that the effect of adrenocortical activity on magnesium excretion is contingent upon the development of changes in urinary sodium and chloride excretion. Withdrawal of adrenocortical hormone from adrenal-deficient patients leads to increased urinary excretion of sodium and magnesium (Table 5; Table 8, line 3), but prevention of the change in sodium excretion by simultaneous restriction of dietary sodium also prevents the steroid-withdrawal effect on magnesium excretion (Table 5; Table 8, line 2).

The possibility cannot be dismissed at once that the various imposed conditions brought about some common physiologic change which in turn influenced alike the urinary excretion of magnesium and of sodium or chloride. It will be necessary first to turn aside and consider the physiologic functions which might most plausibly be thought of as capable of influencing the renal excretion of these 3 ions.

Imposed variation of dietary salt intake might affect urinary magnesium excretion by means of altering endogenous adrenocortical activity. Aldosterone is the principal adrenocortical adrenal hormone requiring consideration in this connection.[2] It is established that aldosterone secretion is stimulated by salt restriction (19, 20); accordingly, the decreased magnesium excretion accompanying salt restriction might be thought to be an aldosterone effect. However, comparison of the data of Table 1 (or Figure 2, line 1) with those of Table 5 (or Table 8, line 2), clearly shows that the mineralocorticoid hormonal effect upon urinary magnesium excretion (aside from effects mediated by change in sodium and chloride excretion) is negligible; when dietary salt and adrenal hormone (including desoxycorticosterone in 4 of 5 instances) were simultaneously withdrawn from severely adrenal-deficient subjects, urinary magnesium decreased exactly as it did in normal subjects subjected to simple salt restriction. Moreover, the magnitude of the change in magnesium excretion was comparable in adrenal-deficient patients (B.L., E.M.(I) and G.D., Table 1) and normal volunteer subjects (M.P. and L.K., Table 1) subjected to comparable salt restriction alone. All these data show that no adrenal hormone can be of much significance,

[2] Leaf and Couter (16) reported what they took to be indications of enhanced glucocorticoid function in volunteers subjected to drastic salt deprivation. Our own observations on circulating eosinophils, nitrogen balance, and urinary excretion of phosphorus, uric acid and potassium in 2 volunteers (who were, however, subjected to less drastic salt restriction) afford no indication that glucocorticoid secretion was stimulated (Table 1 and unpublished data). Measurements of urinary hormone excretion have indicated that salt restriction does not normally stimulate glucocorticoid secretion (17, 18). If dietary salt restriction were affecting urinary magnesium excretion by means of a modest enhancement—no more could be supposed—of glucocorticoid activity, one would expect much larger effects on urinary magnesium than are observed when near-maximal stimulation of glucocorticoid secretion is effected by administration of corticotropin (Table 2).

even in a permissive capacity, in the production of the changes in magnesium excretion observed after salt restriction.

Urine volume, extracellular fluid volume, glomerular filtration rate, and urinary excretion of potassium and organic substances. Changes in glomerular filtration rate, to be more fully discussed subsequently, did not bear a constant relation to changes in urinary magnesium excretion. Urinary excretion of organic substances other than nitrogenous compounds was not measured but seems unlikely to have been of importance in the studies in which dietary salt only was altered. There was also no constant parallelism between the urinary excretions of magnesium and potassium (compare Tables 1 and 5 with Tables 2, 3 and 4).

Urinary magnesium excretion paralleled urine volume in those periods (Tables 2, 3 and 4) in which only a change of adrenocortical activity was under study, but there was no such parallelism in the other periods. Moreover, persons with adequate adrenal function subjected to simple dietary salt restriction excreted an increased volume of urine for at least two days while urinary magnesium excretion was decreasing. It is known that increases in extracellular fluid volume occur promptly under the influence of adrenocortical steroids or adrenocortical stimulation by corticotropin (9, 21), as shown in Tables 2 and 3, and that decreases regularly accompany the development of acute adrenocortical insufficiency (9, 22–25) (Tables 5 and 7). Since urinary magnesium excretion diminished throughout all these periods, the decreases must be presumed to have been unaccompanied by any consistent direction of change in extracellular fluid volume.

To summarize: the parallelism between the rate of urinary excretion of magnesium, sodium and chloride throughout the studies just discussed is unlikely to be fortuitous, could hardly reflect a dependence of sodium and chloride excretion on magnesium excretion, and seems not to represent a joint influence exerted upon the excretion of the 3 ions by imposed or secondarily occurring changes in adrenocortical activity, extracellular fluid volume, urine volume, glomerular filtration rate, or in the excretory rate of other substances. The findings are best explained in terms of a linkage between the rate of renal excretion of magnesium, sodium and chloride such that net magnesium excretion is accelerated or reduced, respectively, as the excretion of sodium and chloride is accelerated or reduced. It is not implied that these relationships would necessarily continue to obtain throughout periods of observation in excess of six days.

Relevant observations by others

In 1952, Haynes, Crawford and DeBakey (26) observed a pattern of diminished urinary magnesium and sodium excretion and increased potassium excretion by patients following surgical procedures. Since this pattern

of electrolyte excretion could be reproduced in other subjects by the administration of adrenocorticotropin, it was thought to be a consequence of increased adrenocortical activity. The observations reported in this communication are entirely in agreement with those of Haynes *et al.*, but permit more precise definition of the nature of the relationship between adrenocortical activity, urinary sodium and chloride excretion, and urinary magnesium excretion.

In contrast, Miller, Falloon and Lloyd recently published observations (27) on the basis of which they concluded that variations in sodium chloride intake did not significantly affect urinary magnesium excretion by human subjects. In 2 of their studies these investigators utilized, on alternating days, one of two diets calculated to be "nearly constant in magnesium" on the basis of data furnished in food tables; the accuracy of this estimation was not given. It seems not unlikely that the difference in the magnesium content of the two diets may have been sufficient to obscure small changes in urinary magnesium excretion referable to changes in urinary sodium chloride excretion. The imposed reduction of daily sodium intake between periods in all their individual studies was 86 mEq. per day, a considerably smaller reduction than was made in our studies; nevertheless, in all 3 of the studies in which dietary magnesium was kept indubitably constant, a small but progressive decrease of urinary magnesium excretion throughout the period of dietary salt restriction was observable, with increased urinary magnesium excretion during the ensuing period of increased salt intake.

The relation between the urinary excretion of sodium chloride and that of magnesium may explain the mechanism of certain drug effects which have been reported as isolated phenomena. The increased urinary output of magnesium which has been observed after administration of mercurial (2) and other diuretics (28, 29) may well be a consequence of the natriuretic and chloruretic properties of these drugs. Tapley (30) noted increased urinary magnesium excretion when *l*-triiodothyronine was administered to patients with myxedema. Since it has been reported by several investigators (31, 32), though not by all, that treatment of myxedematous subjects with thyroid hormones causes an increase in urinary sodium excretion, the reported effect of *l*-triiodothyronine on magnesium excretion can perhaps be explained as a consequence of natriuresis or chloruresis, especially as the urinary excretion of magnesium in hyperthyroidism seems to be normal (33).

In view of the foregoing observations, it appears unlikely that the hypomagnesemia (34, 35) and negative magnesium balance (35) which have been recorded in cases of primary aldosteronism can be ascribed to a direct hormonal affect of aldosterone upon renal magnesium excretion. It seems

probable, as already suggested by others (27), that magnesium waste in that condition is to be ascribed to an intrinsic tubular defect constituting an additional pathophysiologic feature of kaliopenic nephropathy (36). This explanation also accounts for the delayed correction of renal magnesium waste, contrasting with the immediate fall of the hormone-controlled potassium clearance, which has been reported (35) to follow surgical correction of primary aldosteronism.

Renal mechanisms involved in the observed changes of magnesium excretion

The stability of the plasma levels of magnesium (not shown) in all patients except those in whom adrenocortical insufficiency developed was such as to suggest that the observed changes in urinary magnesium excretion were not the consequence of changing plasma levels resulting from events occurring at cell boundaries, in connective tissue or on crystal lattices of bone, but instead represented primary effects upon the renal clearance of magnesium.

In all the experiments of Table 5 and some of those of Table 1, decreases of urinary magnesium excretion were accompanied by falling rates of glomerular filtration and apparent magnesium filtration.[3] Conclusive evidence of a specific effect on net tubular reabsorption of any substance generally requires that the filtration rate of that substance be shown to rise while the urinary excretion of the substance is falling, or vice versa. The periods during which it might be thought that such conditions were fulfilled for magnesium excretion are those presented in Tables 2, 3 and 4. In Patient B.J. (Table 3) the glomerular filtration rate was estimated (Table 7). There can be little question that in the hormone withdrawal period in this patient the increased urinary excretion of magnesium occurred together with a declining glomerular filtration rate. The decline was reflected in a fall of inulin clearance as well as in 24-hour creatinine clearance; the latter increased again during the second control period, indicating a rise in the glomerular filtration rate as urinary magnesium excretion fell. Apparent filtration of magnesium (estimated as inulin clearance multiplied by plasma magnesium concentration) also fell. Since it has been demonstrated repeatedly that administration of cortisone or hydrocortisone, as well as stimulation by means of corticotropin of the secretion of a competent adrenal cortex, reg-

[3] Apparent magnesium filtration rate may be defined as glomerular filtration rate $\times$ serum magnesium concentration $\times k$ (an approximate correction factor for nondiffusible magnesium). A change in the proportion of diffusible magnesium during the experiments would invalidate the use of apparent magnesium filtration as an estimate of true magnesium filtration, and so prevent the estimation of reabsorbed magnesium. There is apparently no information available concerning the effect of adrenal hormones upon the extent of binding of magnesium by plasma proteins. It seems unlikely that alterations of dietary sodium chloride would materially affect the diffusibility of magnesium.

larly results in a marked early increase in the filtration rate (21, 25, 37), it may be assumed that in Patient D.M. (Table 2) and Patient J.V. (Table 4) the decreased urinary excretion of magnesium during the experimental period occurred with a rising glomerular filtration rate, and the increased excretion during the second control period occurred with a falling filtration rate. Apparent magnesium filtration must have paralleled glomerular filtration, in view of the stability of plasma magnesium concentration.

The values for 24-hour creatinine clearance in Subjects L.K. and M.P. (Table 1) indicate that the glomerular filtration rate was little affected by reducing, without drastically restricting, dietary salt. The change in the mean value was rather small in both instances, and in Subject L.K. it was an increase. The data suggest that changes in the filtration rate are not the cause of decreasing urinary magnesium excretion when dietary salt intake is moderately restricted.

On the assumption (not warranted at present) that no change in the diffusibility of plasma magnesium occurred, the data would indicate that, when urinary magnesium excretion is reduced as a result of increasing adrenocortical activity, the reduction is accomplished by a specific increase in the transport of magnesium across the renal tubular epithelium, resulting in increased net tubular magnesium reabsorption; such is known to be the case in respect to sodium and chloride (25). The data on the 2 volunteers subjected to restriction of dietary sodium chloride also suggest increased net tubular reabsorption of magnesium, but here the evidence is not entirely conclusive owing to the difficulty of being certain that some fall in glomerular filtration rate might not have occurred.

Relative importance of sodium versus chloride excretion upon magnesium excretion, and the influence of other ions

The data in Table 6 show that there are conditions under which urinary magnesium excretion may parallel that of chloride rather than sodium excretion. These data do not prove that the chloride ion is more important than the sodium ion in bringing about the changes presented in Tables 1–5; rather, they emphasize that attempts to impose changes in the rate of excretion of a single ion inevitably involve associated changes, the relative importance of which it is difficult to assess. In this instance the imposed conditions must have caused changes in extracellular and perhaps in intracellular pH, and in the rates of excretion, not only of sodium, chloride and bicarbonate ions, but also of hydrogen ion, ammonia, citrate, and perhaps other substances. Collation of all available published data does not establish how many of these many variables are significantly related to urinary magnesium excretion, but suggests that individual ion effects may be important. The opposed effects of alkalinizing and acidifying salts in our

studies and those of others (5, 6) might indicate a causative relationship between change of pH (either of urine or of extracellular fluid) and change in magnesium excretion; and the lack of influence on urinary magnesium of acetazolamide (38), which induces systemic acidosis associated with an alkaline diuresis (39), might further suggest that reduction of pH in extracellular fluid and in urine each independently stimulates urinary magnesium excretion, whereas a rise of pH in either fluid depresses it. Yet the administration of sodium lactate, under conditions different from ours, is said (38) to enhance urinary magnesium excretion, though it raises the pH of both urine and extracellular fluid. These data cannot all readily be reconciled with the supposition that urinary magnesium excretion is influenced *only* by means of acid-base effects. It is even possible that such effects influence urinary magnesium principally by affecting the rate of excretion of individual ions, among which must also be included sulfate (40). Clearly it would be premature to attempt to appraise, on the basis of the available data, the relative individual contributions of sodium and chloride ions to the effects upon urinary magnesium excretion recorded in Tables 1–5.

Renal regulation of potassium and calcium excretion in relation to sodium chloride excretion

When dietary sodium chloride was restricted in persons with adequate adrenocortical function, the urinary excretion of potassium regularly decreased, along with that of sodium, chloride and magnesium (Table 1). The same effect was seen when, along with the salt supplements, replacement steroid therapy was withdrawn from adrenal-deficient subjects (Table 5). On the other hand, administration and withdrawal of larger amount of glucocorticoid compounds, or of corticotropin, induced changes in potassium excretion which reflected the well-known kaliuretic effect of these compounds (Tables 2, 3 and 4) and were opposite in sense to the change in urinary sodium and chloride excretion.

The recorded decreases in urinary potassium in the adrenal-deficient patients (Table 5; and G.D., Table 1) can doubtless be ascribed in part to withdrawal of the kaliuretic effect of the replacement steroids. The falls in mean daily potassium excretion in subjects with adequate adrenocortical function subjected to dietary salt restriction were consistent but sometimes small, and not statistically significant in 5 cases. The evidence linking urinary potassium excretion to urinary sodium chloride excretion is therefore relatively weak. There are, however, data in the literature (41) which also suggest that potassium excretion decreases when dietary sodium chloride is definitely reduced in persons otherwise receiving constant diets, in spite of the fact that sodium restriction enhances the secretion of aldosterone.

The data on calcium excretion (Tables 1 and 3) indicate that the rate of urinary excretion of this ion, like that of magnesium, is linked to the rate of urinary excretion of sodium and chloride. Although the data are not extensive, they are consistent; whether sodium chloride excretion was manipulated by dietary or hormonal means, urinary calcium excretion exhibited sizable parallel changes. The calcium changes could be explained in part (13, 15, 42) but, in view of their magnitude, hardly altogether, as a secondary consequence of the increased excretion of magnesium. Indeed, it is equally possible that the changes in urinary magnesium excretion were, in part or principally, a consequence of the changes in urinary calcium excretion effected by a change in urinary sodium chloride excretion.

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THE TUBULAR REABSORPTION OF PHOSPHATE IN THE DIFFERENTIAL DIAGNOSIS OF METABOLIC BONE DISEASE

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ABSTRACT

The percentage of filtered phosphorus that is reabsorbed by the renal tubules (TRP) was measured in normal subjects and in patients suspected of having metabolic bone disease. When dietary intake of phosphorus before the test was not controlled, the TRP ranged between 77 and 93 per cent in normal subjects and patients without demonstrable disturbances of calcium and phosphorus metabolism. No correlation could be established between the serum phosphorus concentration and the TRP. If the diet contained adequate amounts of phosphorus, the TRP was low in all of the cases of primary hyperparathyroidism that were studied; after phosphorus deprivation, the TRP was often in the normal range. These observations are considered to be consistent with the view that parathyroid hormone lowers the maximal tubular capacity for phosphate reabsorption. The TRP was consistently low in patients with malignant neoplasms complicated by hypercalcemia. The chief usefulness of the TRP test in clinical diagnosis lies in the early diagnosis of hyperparathyroidism when only slight abnormalities of the serum calcium and phosphorus concentration can be detected.

THE plasma concentration of a hormone generally provides the most definitive information concerning the functional state of an endocrine gland. Since such a measurement of the parathyroid hormone has not yet been developed, several indirect indices of parathyroid function have been devised. Of these, Howard's calcium infusion test (1–3) and the renal tubular reabsorption of phosphate (3, 4) showed the greatest initial promise. Recent reports (3, 5) suggest that both tests may yield equivocal or misleading information in the diagnosis of parathyroid dysfunction.

In the present study, the tubular reabsorption of phosphate (TRP) was measured in a wide spectrum of illnesses in which abnormalities of calcium and phosphorus metabolism were suspected. The nonspecificity of the test

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in the differential diagnosis of hypercalcemia was established, and the usefulness of the test was further defined.

MATERIAL AND METHODS

The TRP was measured in 8 normal subjects and in 59 patients with a variety of illnesses. The test was always performed in the morning with the subjects in a post-absorptive state. The ingestion of abundant amounts of water was encouraged during the test period. Urine was collected during a four-hour test period, and venous blood was obtained at the mid-point. Although it is known that the level of phosphorus intake preceding the test influences TRP (4), no effort was made to standardize the phosphorus intake. However, a dietary history was always obtained, and if it was suspected that the diet had been deficient in phosphorus, the test was repeated after the ingestion of a quart of milk daily for three to six days. A quart of milk contains about 1 gram of phosphorus, which approximates the normal intake of this mineral. When the ingestion of milk was contraindicated because of its high calcium content, a mixture of monobasic and dibasic sodium phosphate containing approximately 1 gram of phosphorus was prescribed for three to six days. None of the patients had significant disturbances of acid-base balance, as indicated by a normal concentration of serum bicarbonate. The concentration of serum potassium was normal in all but 1 patient. Since renal insufficiency is known to decrease TRP (6), patients with azotemia were not included in this report.

The concentrations of creatinine and phosphorus were determined in urine and serum by the methods of Hawk, Oser and Summerson (7) and of Gomori (8), respectively. Standard curves were determined every day. Urine specimens were not always accurately timed, and creatinine clearances calculated from the data in the tables therefore do not reflect true filtration rates. However, since the clearances of phosphate and creatinine were determined simultaneously, errors in the timing of collections did not affect the calculated values of TRP.[1]

RESULTS

Normal or high TRP

The data on patients whose TRP was normal or high are listed in Table 1. Of these, 8 were normal physicians and medical students, 23 had renal calculi, and 13 had miscellaneous illnesses.

Normal subjects. The TRP was 85.5 ± 5.4 (mean ± 1 S.D.).

Renal calculi. The TRP was 85.0 ± 3.2. The cause of calculus formation could not be determined in these patients. Hyperparathyroidism, idiopathic hypercalciuria, gout and cystinuria were excluded or rendered very unlikely by the data of the history, physical examination, and the usual laboratory tests.

Since no significant difference could be detected between the values for TRP in normal subjects and the patients with renal calculi, the samples

[1] For a simplified method of calculation of TRP, see Chambers *et al.* (3). TRP (as per cent of filtered phosphate that is reabsorbed)

$$= 100\left(1 - \frac{\text{urine phosphorus} \times \text{serum creatinine}}{\text{serum phosphorus} \times \text{urine creatinine}}\right).$$

TABLE 1. PATIENTS WITH NORMAL OR HIGH TRP

Patient	TRP (%)	mg./100 ml.				Urine vol. (ml.)	Remarks
		Serum P*	Serum Cr*	Urine P	Urine Cr		
	85.5 ±5.4	(mean ±1 S.D.)					8 normal subjects
	85.0 ±3.2	(mean ±1 S.D.					23 patients with renal calculi; no other demonstrable metabolic abnormalities
N.W.	85.1	5.1	1.5	29.2	56.6	350	Renal tubular acidosis; no evidence of osteomalacia
B.C.	91.9	3.4	1.0	2.2	8.1	1240	Renal tubular acidosis; no evidence of osteomalacia
G.F.	83.0	3.8	0.74	65.9	75.2	76	Malabsorption syndrome; serum P as low as 2.2 mg./100 ml.; alkaline phosphatase normal
E.C.	89.8	2.9	1.1	26.4	94.3	200	Possible osteolamacia; serum P as low as 2.6 mg./100 ml.; alkaline phosphatase normal
M.P.	88.5	2.7	0.75	5.8	13.9	640	Malabsorption syndrome with severe osteomalacia; alkaline phosphatase =15 B.U.
M.R.	90.1	4.4	1.0	9.5	22.9	985	Osteoporosis
R.L.	91.5	3.6	1.0	18.5	61.0	1000	Idiopathic hypercalciuria with renal calculi; 3-hr. urine
T.N.	88.5	3.9	1.3	14.3	39.8	740	Idiopathic hypercalciuria with renal calculi
E.K.	90.6	4.2	1.2	64.2	197.8	175	Histiocytosis X
L.D.	94.2	3.7	1.3	31.0	191.0	196	Patient with renal calculi receiving low-P diet; ? glycinuria
L.M.	94.2	3.9	0.8	49.8	172.4	56	Patient with recurrent pancreatitis receiving low-P diet
A.B.	94.4	3.3	1.2	3.4	21.3	1420	Patient with renal calculi receiving a low-P diet
A.Z.	99.5	2.0	0.89	0.20	12.5	1880	Acute alcoholism; potassium depletion

* P = phosphorus. Cr = creatinine.

were combined for the estimation of the normal range. The 95 per cent confidence limits of normal TRP thus obtained were 77 to 93 per cent.

Miscellaneous illnesses. Patients N.W. and B.C. had typical *renal tubular acidosis.* At the time of the test, they did not show any evidence of active osteomalacia or of hypokaliemia. Patients G.F. and E.C. had hypophosphatemia with a normal concentration of serum alkaline phosphatase. Patient M.P., on the other hand, had severe active *osteomalacia.* It is noteworthy that TRP was normal in these 3 patients, although a decrease might have been anticipated owing to secondary hyperparathyroidism. In this connection, Nordin's recent emphasis on the relationship between serum phosphorus concentration and TRP should be mentioned (9). Recalculating the data of Milne, Stanbury and Thomson (10), he noted an inverse relationship between serum phosphorus concentration and TRP in

normal subjects. Such a correlation was not noted in our data. In Figure 1, the regression coefficient approximates zero, and the correlation between serum phosphorus concentration and TRP is not significant.

Effect of the level of phosphorus intake on TRP. This is illustrated by Patients L.D., L.M., A.B. and A.Z. The phosphorus intake was low in these cases and the TRP high. In normal subjects and in patients with renal calculi, the ingestion of one quart of milk daily for three to six days resulted in a slight decrease of TRP. With this dietary regimen, TRP decreased from 85 to 82 per cent (average), but rarely to a value as low as 80 per cent, and never to the critical lower limit of normal.

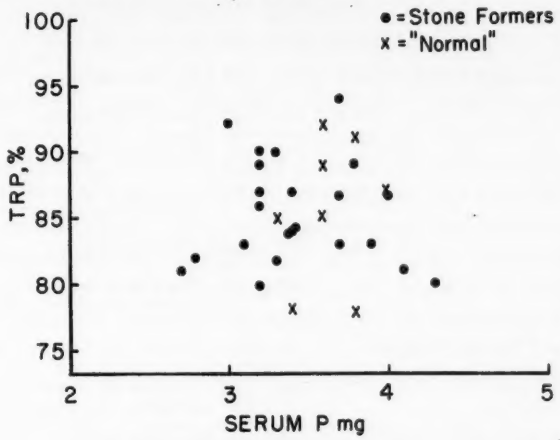

FIG. 1. There was no significant correlation between serum phosphorus concentration (mg./100 ml.) and TRP (%) in normal subjects and patients (stone formers) with renal calculi but without any other demonstrable metabolic abnormality.

Low TRP

The data on patients whose TRP was low are listed in Table 2. Nine had primary hyperparathyroidism, 10 malignant neoplasms, and 4 miscellaneous illnesses.

Hyperparathyroidism. Among the 9 patients listed, the diagnosis was proved in 7 by the removal of one or more adenomas. In the other 2 patients (A.H. and McD.), the findings were completely characteristic of hyperparathyroidism, but surgical confirmation had not been achieved. In 3 of the proved cases (M.K., H.M. and R.S.) and in 1 suspected case (A.H.), the TRP was initially normal but decreased markedly when milk was ingested. Patient M.K. had a very low phosphorus intake because of vomiting associated with pancreatitis, which complicated the hyperpara-

TABLE 2. PATIENTS WITH LOW TRP

Patient	TRP (%)	mg./100 ml.					Urine vol. (ml.)	Remarks
		Serum Ca*	Serum P*	Serum Cr*	Urine P	Urine Cr		
Hyperparathyroidism								
M.B.	68.6	10.1–11.2	2.4	1.2	7.50	11.8	1020	Parathyroid adenoma; low serum P and normal serum calcium
M.K.	86.0	11.8–14.2	1.5	1.0	4.28	19.5	1000	Parathyroid adenoma
	75.3		2.5	0.94	45.3	68.8	400	After 3 days of milk ingestion
	55.0		2.2	1.0	47.2	48.3	298	After 6 days of milk ingestion
E.B.	55.5	14.0–16.5	2.9	1.8	25.7	37.0	209	Parathyroid adenoma
H.M.	85.0	12.8–14.0	2.2	1.1	22.4	76.0	520	2 parathyroid adenomas
	65.5		2.5	1.3	94.0	139.4	238	After milk ingestion
R.S.	78.6	12.3–12.4	2.9	1.2	11.2	21.4	695	Parathyroid adenoma
	61.0		3.1	1.0	25.1	20.8	865	After milk ingestion
D.N.	76.4	10.7–14.4	2.8	1.0	22.4	33.6	490	Parathyroid adenoma
V.S.	71.4	12.1	2.9	1.0	15.9	19.7	870	Parathyroid adenoma
A.H.	82.9	11.5–13.0	2.5	1.1	40.6	101.7	485	Typical history and laboratory findings of hyperparathyroidism; patient refused surgery
	70.7		2.3	1.2	60.1	108.8	290	After milk ingestion
McD.	75.0	12.8–14.1	2.0	1.4	33.9	96.8	250	Typical history and laboratory findings of hyperparathyroidism; inadequate surgical exploration
Malignant Neoplasms								
J.C.	38.1	16.7	3.0	1.5	104.0	84.0	160	Carcinoma of undetermined origin, with liver metastases
E.A.	78.3	12.8–13.2	3.0	0.89	15.1	20.9	765	Hypernephroma
L.P.	68.5	12.4	4.8	0.94	156.0	94.3	187	Hypernephroma
N.I.	52.5	14.0–16.5	3.0	1.7	26.4	30.5	300	Multiple myeloma
C.A.	69.8	10.8–12.6	3.6	1.2	112.6	121.1	87	Chronic lymphatic leukemia
A.C.	68.1	12.8	3.4	1.3	42.3	48.2	475	Carcinoma of lung with bone metastases
L.B.	46.8	14.8–15.8	3.5	1.5	41.6	34.2	265	Carcinoma of lung with bone metastases
N.T.	59.8	9.6–14.0	3.8	1.3	29.1	23.7	920	Carcinoma of undetermined origin, with metastases to pelvic bones
C.H.	73.3	14.1	4.3	1.5	27.2	35.9	165	Multiple myeloma
H.B.	61.0	11.0–13.6	3.0	1.1	40.5	39.8	300	Carcinoma of lung
Miscellaneous								
R.McG.	76.1	9.6–10.3	3.5	1.0	15.5	19.0	1174	Chronic pyelonephritis with stones; right nephrectomy 2 yrs. previously
E.R.	75.9	10.0–11.0	3.1	1.6	39.2	85.7	365	Left nephrectomy 6 yrs. previously for hypernephroma
I.W.	68.3	9.8–10.0	2.1	0.83	55.6	68.4	158	Adult with healed vitamin D-resistant rickets and untreated thyrotoxicosis
A.G.	62.1	9.8–11.0	1.8	0.89	11.4	15.1	1200	Adult with acquired vitamin D-resistant osteomalacia

* Ca = calcium. P = phosphorus. Cr = creatinine.

thyroidism. With a milk regimen, TRP decreased progressively from 86 to 55.0 per cent. Patient H.M. had a low phosphorus intake for several days before the first test because of roentgenologic examinations of the gastrointestinal tract. After drinking milk for four days, TRP decreased from 85.0 to 65.5 per cent. Ingestion of milk effected a decrease of TRP from 78 to 61 per cent in Patient R.S. and from 82.9 to 70.7 per cent in Patient A.H. These responses should be contrasted with those of normal subjects, which have already been described.

In all cases of hyperparathyroidism, hypercalcemia was present at some time, except in Patient M.B. In this case, the level of serum calcium was consistently normal and the level of serum phosphorus low. At operation, no evidence of infarction of the adenoma could be found.

Malignant neoplasms. All 10 of these patients had hypercalcemia. There was a crude negative correlation between concentration of serum calcium and TRP. The serum phosphorus concentration was normal in all but 2 cases.[2] Patients J.C. and H.B. had marked hypophosphatemia on several occasions. Although osseous metastases could not be detected roentgenologically in Patients J.C., C.A. and H.B., widespread visceral involvement was evident in all three. Two patients (C.H. and H.B.), with symptoms that were probably caused by hypercalcemia, were treated with corticosteroids. During this therapy, the serum calcium concentration promptly decreased in one (C.H.) in association with an increase in TRP from 73.3 to 89.5 per cent, but did not change in the other (H.B.), in whom TRP also remained unchanged.

It should be noted that there is a complete overlap of the values for TRP in primary hyperparathyroidism and malignant neoplasms with hypercalcemia.

Miscellaneous illnesses. In the 2 patients who had only *one kidney*, TRP was slightly below the lower limits of normal. Patient I.W. came from a family affected with *vitamin D-resistant rickets.* It is not known whether the low TRP observed was a reflection of this disease or of the complicating thyrotoxicosis. Patient A.G. was of unusual interest and will be reported in greater detail elsewhere; she had nonfamilial vitamin D-resistant osteomalacia that began late in adult life.

DISCUSSION

The existence of a maximal renal tubular reabsorptive capacity for phosphate (TmP) has been impressively documented by several investigators, both in man (11, 12) and in the dog (13–15). Unlike the Tm for glucose, however, TmP varies between wide limits in normal man and is not even a

[2] Normal serum phosphorus concentration in this laboratory is 3.6 ± 0.3 mg./100 ml., with 95 per cent confidence limits of 2.9 to 4.3 mg./100 ml.

consistent value in individual subjects studied repeatedly while receiving comparable intakes of calcium and phosphorus (11, 12).

TmP is apparently influenced by the parathyroid hormone; it is decreased in the presence of this hormone and increased during a deficiency (16). Evidence presented by Nordin and Fraser (17) and confirmed by observations from this laboratory (18) suggests that the level of circulating parathyroid hormone may change very rapidly under certain experimental conditions. If hyperphosphatemia is accepted as a stimulus for parathyroid secretion (4), the variability of TmP in individual patients reported in the literature becomes understandable. The determination of TmP involves the infusion of phosphate and changing levels of serum phosphorus; the latter often do not change reproducibly between experiments and may therefore be associated with variable states of parathyroid function.

Evidence presented in this paper is consistent with the view that the TmP is decreased in hyperparathyroidism. In Figure 2, the regression of reabsorbed phosphorus on filtered phosphorus is plotted for "normal" subjects (healthy persons and patients with renal calculi but no demonstrable metabolic abnormality).[3] The linearity of the relationship indicates that all of the studies were performed well below the TmP for these individual patients. As TmP is approached, the amount of reabsorbed phosphorus should approach a constant value with increasing loads of filtered phosphorus. Although the points representing normal samples fluctuate randomly above and below the calculated line, all but 2 of the points for patients with hyperparathyroidism are to the right of this line, and a few of them are markedly to the right. Although TmP was not determined in these patients, the data in Figure 2 suggest that TRP is lowered in hyperparathyroidism when the filtered load of phosphorus begins to approach the markedly decreased TmP. This explanation is in accord with the observation that TRP may be normal in hyperparathyroidism and decrease only after sufficient quantities of phosphate have been given in the diet. In the presence of phosphate depletion, the amount of filtered phosphorus may be so small as to be far below even the decreased TmP resulting from the hyperparathyroid state. As the amount of filtered phosphorus is increased by dietary means, the TmP is approached and TRP then decreases.

An alternative explanation for the lowered TRP in hyperparathyroidism

[3] The good correlation noted in this figure is, at least in part, artifactual. The reabsorbed phosphorus cannot be determined independently of the filtered phosphorus. Any systematic error tending towards an overestimation of filtered phosphorus would result in a corresponding overestimation of reabsorbed phosphorus. In addition, since TRP is normally 85 per cent, filtered and reabsorbed phosphorus differ from one another by a relatively small quantity. All of these factors may produce a misleadingly good correlation.

may be considered. Parathyroid hormone may affect, not the TmP but the shape of the curve, as TmP is approached ("titration splay"). There is usually a rather sharp change of slope near the Tm. If parathyroid hormone caused this change to take place gradually over a wide range of filtered phosphorus, TRP would be low in hyperparathyroidism. Our data do not permit differentiation between these possibilities. It should be noted, however, that there is experimental evidence concerning the lowering of TmP by parathyroid hormone but none concerning any alteration in the shape of the curve.

Our experience with osteomalacia is inconclusive, since some depression of TRP would be expected as a result of secondary hyperparathyroidism. Of the patients recorded in Table 1, only one (M.P.) had severe active osteomalacia. The filtered phosphorus was very low in this patient, and the

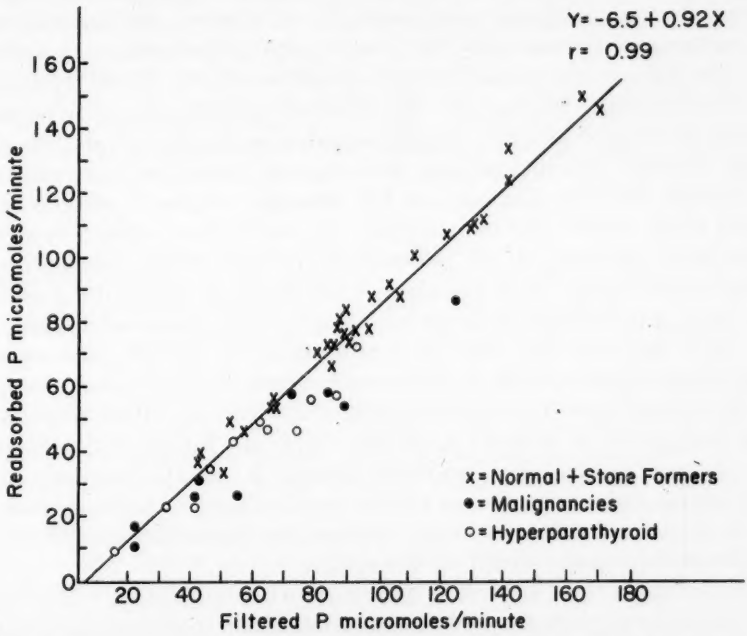

FIG. 2. Regression of reabsorbed phosphorus on filtered phosphorus calculated for normal subjects and patients (stone formers) with renal calculi but without any other demonstrable metabolic abnormality. The studies were made below the TmP, since the function appears to be linear throughout. TmP would be represented by a line parallel to the X-axis at various levels of Y. A lowered TmP in patients with hyperparathyroidism and with malignant tumors is suggested by the fact that points for these patients lie to the right of the line. Note also that TRP in these patients was often normal (points on or near the line) if the filtered phosphorus was very low.

measurements were made before the importance of this variable was fully appreciated. Others have reported a decrease of TRP in osteomalacia (17). The slightly decreased TRP in the 2 patients who had only one kidney may have been attributable to secondary hyperparathyroidism: as the load of filtered phosphorus was decreased, a tendency towards hyperphosphatemia would exist; the parathyroid glands would respond by the secretion of increased amounts of parathyroid hormone and thus establish a moderate phosphaturia with maintenance of a normal serum phosphorus concentration.

In the differential diagnosis of hypercalcemia, simple clinical and laboratory aids generally establish or exclude the diagnosis of sarcoidosis, the milk-alkali syndrome, hypervitaminosis D, hyperthyroidism, multiple myeloma, and acute skeletal atrophy of disuse. The most troublesome diagnostic problem is presented by the differentiation of primary hyperparathyroidism from malignant neoplasms with or without demonstrable skeletal metastases. Occasionally, the primary site of the neoplasm is obscure and the skeletal metastases strongly resemble osteitis fibrosa cystica on roentgenographic examination. The biochemical hallmarks of hyperparathyroidism (hypercalcemia, hypophosphatemia and an elevated level of serum alkaline phosphatase) can be completely mimicked by malignant disease (19, 20, 21). Thomas and his colleagues (5) have previously reported a decrease of TRP in malignant disease, but their studies were complicated by azotemia in all patients except one. Renal insufficiency is known to decrease TRP in the absence of malignant disease (6). Azotemia was not a complicating factor in our patients with malignant tumors, yet TRP was markedly decreased. It is of considerable interest that a malignant tumor may elaborate a substance or substances that closely resemble the physiologic activity of a normal endocrine secretion. Bone biopsies are now being made in patients harboring malignant tumors and presenting with hypercalcemia and hypophosphatemia. If the characteristic histologic changes of osteitis fibrosa cystica can be found in some of these patients, the case for the similarity between the malignant secretions and parathyroid hormone would be strengthened.

The failure of the TRP test to differentiate between primary hyperparathyroidism and malignant tumors associated with hypercalcemia decisively detracts from its usefulness in clinical diagnosis. The chief merit of the test appears to lie in the detection of early hyperparathyroidism, particularly when the concentration of serum calcium is normal or only slightly increased (M.B., Table 2). In the experience of some observers, this form of hyperparathyroidism is not uncommon (22). Our limited experience is in accord with that of Goldman *et al.* (23) and of Nordin (9) in that all pa-

tients with primary hyperparathyroidism studied so far have had a low TRP. Investigators who have reported a normal TRP in cases of primary hyperparathyroidism may have studied patients whose intake of phosphorus had been low. At present, the consistent finding of a normal TRP after adequate dietary preparation would argue strongly against a diagnosis of primary hyperparathyroidism.

Addendum

Since this article was submitted for publication, a patient has been studied whose TRP remained normal with high levels of phosphate intake. The findings were otherwise consistent with primary hyperparathyroidism, and a parathyroid adenoma was found at operation.

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THE RENAL CLEARANCE OF PLASMA CORTISOL

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ABSTRACT

Renal clearance of plasma cortisol was studied by measuring total and ultrafiltrable plasma cortisol simultaneously with glomerular filtration rate and urinary cortisol excretion. The findings were consistent with the interpretation that at normal plasma levels, 80–90 per cent of the filtered cortisol is reabsorbed by the renal tubules.

SINCE cortisol is a normal constituent of human urine, it is cleared from the plasma by the kidney. The renal mechanisms involved have not been elucidated. This study explores the relationships between plasma cortisol, plasma ultrafiltrable cortisol, urinary cortisol, and glomerular filtration rate (GFR).

MATERIALS AND METHODS

Cortisol in plasma, plasma ultrafiltrate, and urine was measured by an isotope derivative dilution procedure following methods developed by Dr. R. E. Peterson.[1] Urinary cortisol was measured also by an isotope dilution method (1) utilizing fluorometry and double paper chromatography. Ultrafiltration of 10–15 ml. samples of plasma was carried out by the method of Toribara (2). The clearance of "alkali-stable" inulin was measured by the method of Walser *et al.* (3), and of creatinine by the method of Bonsnes and Taussky (4). Standard clearance techniques were used, except that the subjects were not catheterized. For all but two of the studies, intravenous loading solutions were used to establish diuresis.

Double isotope derivative method for determination of cortisol

General. Dried dichloromethane extracts of plasma, ultrafiltrate, and urine were acetylated with acetic anhydride-H^3. In most of the studies a measured amount of cortisol-21-acetate-C^{14} (1500–2000 DPM) was added to the acetylated samples. (In the other studies cortisol-4-C^{14} (0.32 μg., 2850 DPM) dissolved in 50 μl. of ethanol was added

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[1] We gratefully acknowledge the advice and assistance of Dr. Peterson and Dr. Bernard Kliman.

directly to the samples before they were extracted.)[2] The doubly labeled acetates were then purified by paper chromatography. After chromatography in two systems, the cortisol-21-acetate was oxidized to cortisone-21-acetate. After a final chromatography, the cortisone acetate zone was eluted and counted for carbon^{14} and tritium in a liquid scintillation counter.[3] The carbon^{14} count gives the amount of indicator lost during the purification. Knowing the yield of tritium radioactivity and the specific activity of the tritium-labeled acetic anhydride, the cortisol content of the original sample can be calculated. An additional correction for losses incurred before adding the indicator was necessary when cortisol-21-acetate-C^{14} was used. Recovery studies with comparable known amounts of cortisol gave 60–65 per cent yields in the steps up to acetylation.

Extraction. To each 1 ml. of plasma or 5–10 ml. of ultrafiltrate was added 0.1 ml. of 1 *N* NaOH. The mixture was extracted twice with 5 times its volume of dichloromethane, which had been purified by passage through silica gel (5). The combined extracts were washed twice with 1 ml. of water. The final wash should be nearly neutral (pH 6.8–7.2). Urine (10 ml.) was extracted with 50 and 30 ml. portions of dichloromethane. The combined extracts were washed with 10 ml. of 0.1 *N* NaOH (the wash must be tested to insure that it is alkaline), 10 ml. of 1 per cent acetic acid (the wash must be acidic), and 10 ml. of water (final pH is near neutrality). The extracts were dried, transferred to small glass-stoppered centrifuge tubes with ethanol, and evaporated with final drying under vacuum in a desiccator.

Acetylation. The dried residue dissolved in 20 μl. of dry pyridine was treated with 25 μl. of a 10 per cent solution of acetic anhydride-H^3 in sodium-dried benzene. The stoppered tubes were sealed with pyridine and heated at 37° C. for twenty-four hours. Ethanol (0.1 ml.) was then added to each tube (cortisol-21-acetate-C^{14} when used as the indicator, was introduced at this point dissolved in the ethanol), followed by 1 ml. of water, and 5 ml. of dichloromethane. The tubes were stoppered and shaken, the aqueous layer was separated, and the dichloromethane solution was washed with 1 ml. of water and evaporated to dryness.

Chromatography. System I: Cyclohexane 100, dioxane 100, methanol 50, water 25; 16-hour run. System II: Cyclohexane 50, benzene 100, methanol 100, water 20; 5-hour run. System III: Carbon tetrachloride 100, methanol 100, water 25; 10-hour run.

Whatman No. 1 paper was used. About 10 μg. of nonradioactive cortisol acetate was added to the samples before chromatography in order to permit visual localization of the samples under ultraviolet illumination. The zones were eluted by covering them with methanol in test tubes.

The dried eluates from the second chromatography were treated with 0.1 ml. of 0.2 per cent chromium trioxide in glacial acetic acid (purified by distillation over $KMnO_4$) After five minutes at room temperature, 0.5 ml. of ethanol and 1.5 ml. of water were added. The mixture was then extracted with 8 ml. of dichloromethane and the extract washed with 1.5 ml. of water and dried. This procedure converts cortisol acetate to cortisone acetate[4] for the final chromatography.

The cortisone-21-acetate zone from the final chromatography was eluted directly into counting vials and the solution evaporated. To the residue dissolved in 0.2 ml. of

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[2] We wish to thank the Endocrine Study Section of the National Institutes of Health for supplying the labeled cortisol.

[3] Tri-Carb Counter, Packard Instrument Co., LaGrange, Illinois.

[4] We are indebted to Dr. Peterson for the details of this oxidation procedure.

ethanol was added 5 ml. of phosphor solution.[5] Carbon^{14} and tritium were counted by the screening method (for nonquenching samples) (6). With our instrument, photomultiplier voltage (HV) settings 3 and 4 (820–900v) were effective at different times in screening out tritium counts, and carbon^{14} was counted alone (discriminator setting 10–25 v). At HV setting 10 (1390 v) both isotopes were counted simultaneously (discriminator setting 10–∞). An amount of cortisol-21-acetate-C^{14} (or cortisol-4-C^{14}) identical with the amount added to the samples was counted at the same time. After subtracting the appropriate backgrounds:

$$\text{Micrograms cortisol in sample} = \text{CPM } H^3 \text{ in sample} \times \frac{100}{\%\text{ recovery}} \times \frac{1}{\text{sp. act. of acetic anhydride-}H^3 \text{ in CPM}/\mu\text{g. cortisol}}$$

where:

$$\text{CPM } H^3 \text{ in sample} = \text{CPM @ HV 10} - (K \cdot \text{CPM @ HV 4})$$

$$K = \frac{\text{CPM @ HV 10}}{\text{CPM @ HV 4}} \text{ (of pure } C^{14} \text{ sample)}$$

$$\%\text{ Recovery} = \frac{\text{CPM @ HV 4 (sample)}}{\text{CPM @ HV 4 (added cortisol acetate-}C^{14})}.$$

For runs using cortisol-4-C^{14}, K was obtained as follows: duplicate samples of cortisol-4-C^{14} were acetylated with the same lot of acetic anhydride-H^3, and chromatographed in the three systems as described. From the final cortisone-4-C^{14}, 21-acetate-H^3 zone,

$$K = \frac{\text{CPM @ HV 10}}{\text{CPM @ HV 4}}$$

$$\%\text{ Recovery} = \frac{\text{CPM @ HV 4 (sample)}}{\text{CPM @ HV 4 (added cortisol-4-}C^{14})}.$$

Areas equivalent to the cortisone-21-acetate zone but about 1 cm. proximally and distally were cut out and eluted. These gave counting rates no greater than background at HV 3 or 4. At HV 10 these zones were usually below 10 per cent of the activity of the cortisone-21-acetate zone but occasionally were as high as 15 per cent. The proximal zone usually showed twice the radioactivity of the distal zone. An average of the proximal and distal zone counts was subtracted from the total count of the cortisone-21-acetate zone at HV 10.

Acetic anhydride-H^3 (200–400 mC./mM.)[6] was diluted with 2 volumes of purified acetic anhydride and made up to a 10 per cent solution by volume in sodium-dried benzene. The reagent was standardized by acetylating duplicate 500-μg. portions of cortisol as described previously. The mixture was chromatographed in System I on alcohol-washed paper, the cortisol-21-acetate zone and two equal zones above and below were eluted with ethanol. The eluates were made up to a volume of 15 ml. with ethanol, and 0.1-ml. aliquots were counted at photomultiplier setting 10 as described. A small

[5] The solution contains 0.4% of 2,5-diphenyloxazole and 0.004% of 1,4-di[2-(5-phenyloxazolyl)] benzene (Pilot Chemical Co., Waltham, Massachusetts) in reagent-grade toluene.

[6] New England Nuclear Co., Boston, Massachusetts.

correction (about 2 per cent) was applied by subtracting the average of the counts of the adjoining zones from the cortisol-21-acetate zone. The cortisol-21-acetate content of the eluate was measured by ultraviolet absorbance at 242 mμ. (Beckman Model DU spectrophotometer) and fluorometry (1) of approximately 0.5-μg. aliquots, using appropriate cortisol-21-acetate reference standards. The count per minute per μg. of cortisol was thus obtained for each lot of acetic anhydride-H^3.

Urinary cortisol measured by fluorometry

To 30 ml. of urine was added 0.42 μg. (about 2400 CPM at 62 per cent efficiency) of cortisol-4-C^{14} in 250 μl. of ethanol. The mixture was then extracted once with 300 ml. of dichloromethane and the extract was washed as described for urine specimens.

Chromatography: System I F1: Benzene 100, methanol 50, water 25; 22-hour run. System II F1: Toluene 100, methanol 70, water 30; 14-hour run.

The Whatman No. 1 paper was washed with ethanol, then rewashed immediately before use. The cortisol zone from System II F1 was eluted as described previously and analyzed fluorometrically (1). Fluoresence was measured in a Farrand fluorometer using the following filter combinations: primary, 470 mμ. interference and No. 5113 Corning glass filters; secondary, 540 mμ interference and No. 3486 Corning glass filters. An aliquot of the eluate was counted at HV 6 in the liquid scintillation counter (discriminator setting, 10–100 v). From the specific activity of the sample, and the amount and specific activity of the added tracer, the quantity of cortisol in the sample is calculated as described. Paper blanks cut from zones adjoining the cortisol spot showed fluoresence equivalent to 0.05–0.08 μg. of cortisol.

RESULTS

Table 1 records data from eight studies in 2 normal subjects and 2 patients with cirrhosis of the liver. Plasma cortisol levels determined by the described method were very slightly lower than those found by the chemical methods. Ultrafiltrable cortisol ranged from 5–22 per cent of the plasma level. Ultrafiltrable cortisol has been shown to be approximately 10 per cent (from 5 to 20 per cent) of the plasma cortisol within the physiologic range (7, 8).

From 82 to 92 per cent of the filtered cortisol was reabsorbed in 10 of the 13 individual clearance studies. There was no direct correlation between cortisol excretion and urine flow. When the GFR was lowered by sodium depletion of a normal subject and by paracentesis and sodium depletion of a cirrhotic patient, there was no correlation between cortisol excretion and GFR.

Cortisol loading of Patient M.G. (study 2) raised the ultrafiltrable cortisol from 11 per cent to 30 per cent of the plasma level. A comparable rise in the percentage of ultrafiltrable cortisol is also found in *in vitro* studies when plasma cortisol concentration is increased to 25–30 μg. per 100 ml. or higher (8). A 14-fold increase in filtered cortisol was accompanied by a 67-fold increase in excreted cortisol. This emphasizes the importance of protein binding for conserving cortisol by reducing the amount available for filtration.

TABLE 1. CLEARANCE OF CORTISOL FROM PLASMA

Two sets of data in studies 1 and 2 on D.R., in study 2 on R.C., and in study 1 on M.P. are from analysis of urine specimens collected during two successive periods.

Subject	Study No.	Cortisol (μg./100 ml.)			Clearance* (ml./min.)	GFR** (ml./min.)	Urine vol. (ml./min.)	Cortisol (μg./min.)			Filtered cortisol reabsorbed (%)	Cortisol excretion (μg./24 hrs.)
		Plasma	Ultrafiltrate	Urine				Filtered†	Excreted	Reabsorbed‡		
D.R., ♀, 20 yrs., normal	1	4.6	0.7	2.7	4	90(Cr)	7.7	0.6	0.2	0.4	67	288
				1.1	2	100(Cr)	9.4	0.7	0.1	0.6	86	144
	2	3.2	0.7	1.1	3	103(Cr)	12.3	0.7	0.1	0.6	86	144
				0.8	2	94(Cr)	7.8	0.7	0.06	0.64	91	86
R.C., ♂, 24 yrs., normal	1	4.0	0.3	1.0	2	147(In)	5.9	0.4	0.06	0.34	85	86
	2	3.9	0.2	0.6	2	127(In)§	9.7	0.3	0.06	0.24	80	86
				0.5	1	130(In)§	7.8	0.3	0.04	0.26	87	58
M.P., ♂, 50 yrs., cirrhosis	1	10.6	1.3	11.2	4	71(Cr)	3.2	0.9	0.39	0.51	57	562
				38.0	4	71(Cr)	1.0‖	0.9	0.38	0.52	58	548
	2	11.2	1.3	6.7	1	86(Cr)	2.2	1.1	0.15	0.95	86	216
M.G., ♂, 45 yrs., cirrhosis	1	4.2	0.4	11.8	2	130(In)	0.7¶	0.5	0.09	0.41	82	130
				7.9	1				0.06***	0.44	88	86
	2	9.2	1.0	23.3	2	90(In)§	0.7#	0.9	0.17	0.83	92	245
		43.7****	13.1	16.1	27	95(In)§	7.2††	12.5	11.6***	0.9	7.2	—

* Plasma cortisol clearance (ml./min.) = excreted cortisol (μg./min.)/plasma cortisol (μg./ml.).
** Measured as clearance of inulin (In) or creatinine (Cr).
† Filtered cortisol = ultrafiltrable cortisol × GFR.
‡ Reabsorbed cortisol = filtered cortisol minus excreted cortisol.
§ Inulin clearance lowered by sodium depletion.
‖ Decreasing urine flow following loading.
¶ No loading; urine specimen collected during morning.
# Control specimen before loading.
*** Analyzed by fluorometry.
**** 42.1 μg./100 ml. when analyzed by fluorometry.
†† Collected during final 15 minutes of infusion of 200 mg. of cortisol in 1000 ml. of 4% fructose during 1¼ hours.

In the last column of Table 1 are the values for 24-hour excretion of cortisol, calculated on the basis of the excretion rates observed during these studies, which were performed between 10:00 A.M. and 1:00 P.M. The 24-hour urine specimens from 2 normal women had cortisol contents of 74–101 micrograms. This agrees well with the calculated range of excretion in Table 1, which should be near the maximum 24-hour amount, since the specimens were collected during the period of higher range in the diurnal variation of plasma cortisol concentration.

In Table 2 are data obtained by analyzing standard solutions for known amounts of cortisol at various times.

DISCUSSION

It is postulated that the nonprotein-bound fraction of plasma cortisol is available for filtration at the glomeruli, and that the concentration of cortisol in the glomerular filtrate is identical with its concentration in

TABLE 2. ANALYSIS OF SAMPLES OF STANDARD SOLUTIONS CONTAINING KNOWN AMOUNTS OF CORTISOL (ISOTOPE DERIVATIVE DILUTION)

Amount of cortisol known to be in sample (μg.)	Amount of cortisol found (μg.)	Recovery of cortisol* (%)
0.055	0.047	62.7
0.055	0.045	52.5
0.055	0.048	28.8
0.055	0.045	45.8
0.110	0.089	50.4
0.110	0.090	41.9

$$* \% \text{ Recovery} = \frac{\text{CPM @ HV 4 (sample)}}{\text{CPM @ HV 4 (added cortisol-4-C}^{14})}$$

plasma ultrafiltrate. Since the physical process of ultrafiltration appears to follow the same laws at the glomeruli as at other membranes (9a), the nonprotein-bound cortisol level measured directly *in vitro* may be assumed to be the same as that produced by glomerular ultrafiltration. Nonprotein-bound cortisol would appear to be the physiologically active fraction of plasma cortisol (7, 8). Thus, it is reasonable to expect that the body economy would include a renal mechanism for maintaining the plasma level of this important hormone, and that only a fraction of the filtered cortisol would appear in the urine. However, this 80–90 per cent renal tubular reabsorption is *not* an important factor in reducing adrenal cortisol production. Assuming a plasma cortisol level of 10 μg. per 100 ml., with 1 μg. per 100 ml. as the ultrafiltrable fraction, then at a GFR of 100 ml. per minute only 1440 μg. or 1.44 mg. would be excreted per day.[7] This would amount to only 7 per cent of a total daily cortisol production of 20 mg.

The calculated 24-hour urinary cortisol excretion (Table 1, last column) is interesting in view of the fact that 99 per cent of the glomerular filtrate is reabsorbed under conditions of normal hydration (9b). If reabsorption of cortisol were to follow this pattern, only 14 μg. per day would be excreted. However, 4 to 20 times this quantity appears to be excreted under the conditions of hydration employed here. A similar rate of excretion was also observed in the 2 subjects in a normal state of hydration, and has also been reported by other investigators (10, review). Thus, although cortisol readily penetrates the body cells, there is much less reabsorption of it by the tubules than there would be, were cortisol to diffuse back passively with the bulk of the glomerular filtrate. It does not appear to be necessary to postulate tubular secretion of cortisol to explain the fact that there is not tubular reabsorption of 99 per cent of the filtered cortisol. Active renal

[7] The effect of diurnal variation in the plasma cortisol level is not considered.

tubular reabsorption of filtered cortisol is more consistent with current concepts of renal physiology. This process would appear to be operating near the Tm level, according to the single study with exogenous cortisol loading.

The isotopic method achieved the sensitivity required for analyzing the ultrafiltrates. Surprisingly enough, this rather elaborate technique did not invariably produce reliable data. Sometimes several samples from the same subject showed very high values, even though the control known samples in the same run were analyzed with accuracy and precision. When found, these high values pertained to all samples from that subject, and were observed even when the areas proximal and distal to the cortisone-acetate spot of the final chromatogram showed low tritium counts. Occasionally urine samples yielded high values which were also associated with high activity in the blank spots.

The chief disadvantage of the double isotope method, when cortisol-4-C^{14} is added directly to the sample, is the low specific activity of the available cortisol. Since one is limited in the total amount of activity that can be added (the total quantity of added cortisol should be a fraction of the amount present), the counting errors for C^{14} become large, and even a slight degree of interference by H^3 becomes significant. When cortisol-21-acetate-C^{14} is added after acetylation, the amount can be adjusted so that counting errors are minimal for the size of the samples involved.

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ESTROGEN EXCRETION IN BREAST CANCER PATIENTS BEFORE AND AFTER OVARIAN IRRADIATION AND OOPHORECTOMY

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ABSTRACT

The urinary excretion of estrone, estradiol-17β and estriol was estimated in 96-hour specimens of urine collected from each of 17 premenopausal breast cancer patients on 5 occasions: (a) during the first part of the menstrual cycle (follicular phase), (b) during the second part of the cycle (luteal phase), (c) two months following ovarian irradiation, (d) four months following ovarian irradiation, and (e) two months following bilateral oophorectomy. The greatest variation in the amount of urinary estrogen seemed to depend on ovulation and corpus luteum formation. Ovarian irradiation reduced significantly the urinary excretion of total estrogen (*i.e.*, the sum of estrone, estradiol-17β and estriol) below that in the follicular phase, and resulted in a marked decrease in the ratio of urinary estriol to estrone+estradiol-17β. Bilateral oophorectomy, performed five months following ovarian irradiation, did not result in any further decrease in urinary estrogen excretion.

ACCORDING to current concepts it is generally believed that estrogens stimulate the malignant growth of metastatic breast cancer in premenopausal women. Consequently, the principal aim of present therapeutic efforts is to reduce, or possibly eliminate endogenous estrogen production. Such therapeutic measures include ovarian irradiation (1), oophorectomy (2, 3), oophorectomy combined with adrenalectomy (4–6), hypophysectomy (7, 8), or the destruction of the pituitary gland by irradiation (9–11).

The recent development of reliable chemical methods for the estimation of small amounts of urinary estrone, estradiol-17β and estriol (12, 13) seems to offer a possible means for the assessment of the relative effectiveness of the different therapeutic measures in reducing endogenous estrogen excretion. In this way a possible correlation between urinary estrogen excretion on the one hand, and the therapeutic results on the other hand, could also be studied.

In this paper we report on the effect of ovarian irradiation and bilateral oophorectomy on the urinary excretion of estrone, estradiol-17β and estriol

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in 17 premenopausal women with breast cancer. The same sequence of treatment was employed in each patient and at exactly the same intervals. Thus this study was planned in a form with a completely balanced design in order to make a proper statistical evaluation possible.

MATERIAL AND METHODS

Clinical material

Seventeen premenopausal women with breast cancer were studied. The relevant clinical data are summarized in Table 1.

All patients had normal menstrual cycles when admitted to Radiumhemmet. All of them had previously been treated with radical mastectomy and local irradiation. In 14 of the 17, histologically verified axillary metastases were present. All patients were examined clinically at least every third month. Survival time was registered until April 1, 1959. As may be seen in Table 1, 11 of the 17 patients were alive at this time. Adrenalectomy or hypophysectomy was performed in all cases showing a rapid progression of cancer.[1] Since these operations obviously influence survival time in two different ways, the survival time following each operation is also indicated in Table 1. At the time of writing this report all patients not subjected to adrenalectomy or hypophysectomy are living without clinical signs of metastases. One living hypophysectomized patient (No. 16) has distant metastases one year following operation.

Plan of study

Urine was collected from each patient in 96-hour specimens, on 5 occasions. The first specimen designated as *F* (follicular phase) was collected from the second through the fifth days of the menstrual cycle, calculated from the first day of bleeding. The second specimen, designated as *L* (luteal phase), was collected in the second part of the cycle and represented urine passed on the eighteenth through the twenty-first days of the cycle. Thus the term "luteal-phase urine" will be used in a loose sense in this paper to denote urine passed on the eighteenth through the twenty-first days of the cycle, without any further evidence of a previous ovulation. Since, however, all patients had normal menstrual cycles, and furthermore since the frequency of anovulatory cycles among women seen in Swedish clinics does not exceed 3 to 5 per cent (14, 15), there is a fairly high probability that the majority of these urine specimens represented luteal-phase excretion. After this second sample had been collected, ovarian irradiation was carried out on three consecutive days, irradiating two anterior fields (each 12×12 cm.) and one posterior field (20×20 cm). The physical conditions were: focus-skin distance (FSD), 50 to 60 cm.; filter, 0.5 mm. Cu+1.0 mm. Al; half-value layer, 0.7 mm. Cu; a skin dose of 700 r was given in a single dose on each field. The active dose for each ovary varied from 550 to 650 r (40–45 per cent of the skin dose), dependent on the particular anatomic conditions in the patient. Two months following irradiation a third 96-hour specimen of urine was collected (X_1, *i.e.*, the first post-X-ray specimen). Four months after ovarian irradiation another sample was collected (X_2, *i.e.*, the second post-X-ray sample). Within one month following this last urine collection, bilateral oophorectomy was performed in all patients; thus all 17 women were ovariectomized within five months

[1] We are indebted to Professor J. Hellström, Karolinska sjukhuset, for performing the adrenalectomies and to Professor H. Olivecrona, Serafimerlasarettet, for performing the hypophysectomies.

TABLE 1. SOME CLINICAL DATA ON THE 17 WOMEN WITH BREAST CANCER*

Patient No.	Age (yrs.)	Previous deliveries	Interval between first symptoms and first treatment (days)	Interval between first treatment and ovarian irradiation (days)	Interval between ovarian irradiation and consecutive menstrual bleeding(s) (days)	Subjective menopausal symptoms following ovarian irradiation	Survival-time from first symptom (mos.)	Interval between oophorectomy and next therapeutic measure	Survival-time after adrenalectomy or hypophysectomy
1	44	1	48	79	30	0	>43	—	
2	43	6	540	332	21 52	+ +	50	11 mos.—adrenalectomy	3 mos.
3	42	0	54	117	—	+	29	17 mos.—adrenalectomy	3 days
4	36	1	?	91	20 41 51	+ + +	>41	—	
5	48	2	45	53	8	0	>37	—	
6	37	2	44	109	?	?	18	6 mos.—hypophysectomy	3 mos.
7	44	2	14	51	0	0	>35	—	
8	48	0	30	37	0	+	24	10 mos.—adrenalectomy	7 mos.
9	35	0	186	210	5	+	>41	—	
10	45	2	45	378	14	+	>45	—	
11	35	0	360	91	28 56	+	>43	—	
12	39	2	30	83	24 55	+	13	4 mos.—adrenalectomy	9 days
13	48	0	60	53	7 35	0	>33	—	
14	49	0	30	354	0	0	>41	—	
15	41	1	570	120	21 45	0	57	—	
16	47	0	8	255	0	0	>48	8 mos.—hypophysectomy	12 mos.
17	38	3	90	117	18	0	>33	—	

* In all except Patients 9, 10 and 14, axillary metastases were present, as verified by histopathologic examination.

following ovarian irradiation. The fifth and last 96-hour urine specimen, designated as *S* (surgical castration), was collected two months following bilateral oophorectomy.

Method of assay

Although at least a dozen estrogens are known to occur in human tissues and body fluids (16), for the time being reliable assay methods are available only for the separate estimation of estrone, estardiol-17β and estriol (12, 13, 17). Thus the urinary estrogen analyses reported in this paper had to be restricted to the estimation of these 3 "classic" estrogens. It should be pointed out, however, that in nonpregnant conditions these 3 estrogens seem to be the most important ones, at least from the quantitative point of view (18). The chemical method of Brown (12) as modified by Diczfalusy and Westman (19) and Brown *et al.* (17) was used throughout this study. The former modification differs from the original technique in the deactivation of the alumina and in the composition of solvents used in the chromatography, whereas the latter modification involves the incorporation of a saponification step, devised originally by Bauld (13). The color correction equation of Allen (20) was used. Experimental verification of the assumption underlying the use of this equation in the Brown method has been published elsewhere (21). All estimations were carried out in duplicate.

Reliability of the method employed

In accordance with its usage in previous communications (22–25), the term *reliability* will be used in a general sense to indicate the 4 concepts of accuracy, precision, sensitivity and specificity.

Accuracy. Since the exact chemical nature of urinary "conjugated" estrogens is incompletely known at present, most workers restrict the assessment of accuracy to recovery experiments in which "free" estrogens are added to urine specimens subjected to hydrolysis (*cf.* 13, 19, 23, 26). It is realized that this expedient is only of limited value, since it will not reveal any losses occurring during the hydrolysis step. In the present study, 38 recovery experiments were carried out by 8 workers, adding 10-μg. to 20-μg. amounts of each estrogen to 24-hour urine specimens. The mean percentage recoveries and their standard deviations (s) were: estrone, 83 ± 8.7; estradiol-17β, 83 ± 9.9; estriol, 79 ± 9.9. These data are in agreement with those reported by other workers using the Brown methods (12, 19, 23, 26).

Precision. The precision of the method was assessed by calculating an estimate of the standard deviation (s) from the difference between the two results of duplicate determinations in a series of assays, according to the formula $s = \sqrt{S(d^2)/2N}$ where d is the difference between duplicates and N is the number of pairs of duplicate analyses performed (27, 28). It should be pointed out that a reliable estimate of s ought to be based on a reasonably great number of duplicates (30, or more).

In Table 2 the values obtained in this laboratory on 24-hour specimens during the past two years by 8 technicians are compared with some of those reported in the literature by the use of the "Edinburgh" (Kober) methods. Estimates of the standard deviation calculated from the 84 duplicate analyses carried out on 96-hour specimens collected from the breast cancer cases of the present study are also shown. Two estimates of estrone and 6 of estriol were higher than 40 μg. per 96 hours; these values were not included.

It appears from the data of Table 2, that the estimates obtained by 8 technicians in this laboratory are in good agreement with those reported by Bauld *et al.* (29), but that they are somewhat less favorable than the values reported by Brown *et al.* (23). The explanation may perhaps be sought in the fact that the values reported by Brown *et al.*

TABLE 2. ESTIMATES OF STANDARD DEVIATIONS (s) CALCULATED FROM THE PRECISION DATA REPORTED FROM DIFFERENT LABORATORIES

E_1 = estrone; E_2 = estradiol-17β; and E_3 = estriol. Figures in brackets indicate the number of pairs of duplicate analyses.

Author	*Range, 0.0–4.9 μg./24 hrs.*			*Range, 5.0–40.0 μg./24 hrs.*		
	E_1	E_2	E_3	E_1	E_2	E_3
Brown *et al.* (23)	0.35 [286]	0.33 [395]	0.46 [248]	0.37 [299]	0.45 [96]	0.60 [295]
Bauld *et al.* (29)*		0.41 [60]	0.55 [115]	0.93 [101]	0.60† [45]	1.09 [140]
Present study ("normal" subjects)	0.35 [93]	0.57 [132]	0.58 [104]	1.19 [82]	0.99† [49]	1.40 [98]
	0.0–4.9 μg./96 hrs.			*5.0–40.0 μg./96 hrs.*		
Present study (breast cancer cases)	0.62‡ [14]	0.37‡ [40]	0.88‡ [28]	1.49 [68]	1.57 [44]	1.63 [50]

* Some of these data were obtained as a personal communication from the late Dr. W. S. Bauld.

† No values between 20 and 40 μg./24 hrs.

‡ Includes a fairly large number of zero values.

were obtained by highly skilled workers, whereas those obtained in this laboratory probably reflect the over-all precision which can easily be obtained by the average laboratory technician.

Sensitivity. The sensitivity of the method can be calculated from the estimates of s at low levels (0.0–4.9 μg. per 24 hours as $t.s./\sqrt{N}$. (23). Values calculated from the data of Table 2 are summarized in Table 3.

From the data of Table 3 it seems justifiable to conclude that the estimation of urinary estrogens at levels as low as 1.0 μg. per 24 hours may already have some qualitative significance. It is of interest that also in the breast cancer series, when results were calculated as μg. per 96 hours, the amounts of estrogen (around 1.5–2.0 μg. per 96 hours) were already distinguishable from zero. Since values at or just below the sensitivity of the method may be as close to the "true" value as to zero, a few such low values obtained in the analyses have also been included in Table 4.

As far as the "quantitative" sensitivity of the method is concerned, if it is specified that the percentage error should not be more than 25 per cent ($P = 0.01$), the smallest amount of estrogen (μg. per 24 hours) which can be measured with this accuracy in duplicate determinations can be calculated from the data of Brown *et al.* (23) at 2.5 μg. of estrone, 2.4 μg. of estradiol-17β and 3.3 μg. of estriol. A similar calculation for our series (μg. per 24 hours) gives 2.5 μg. of estrone, 4.2 μg. of estradiol-17β and 4.2 μg. of estriol.

Specificity. The evidence for the specificity of the original Brown-method has been discussed in earlier papers (21, 23, 30–32). In the great majority of urine specimens this method yielded reliable estimates of the estrogen concentration. However, it was found that excretion products of certain adrenocortical steroids (*e.g.*, cortisone) as well as those of a few aperients such as senna, phenolphthalein, cascara and the sedative meprobamate (2-methyl-2-propyl-propane-1:3-diol dicarbamate) interfered with the method (17). Even the administration of stilbestrol, or of ethinylestradiol interfered with the estimates. The incorporation of the saponification step increased further the specificity of the method and eliminated completely the interference caused by cortisone, meprobamate, cascara and phenolphthalein, but not that caused by senna or synthetic estrogens. Consequently Bulbrook *et al.* (33) demonstrated close agreement between the results of bioassays and of chemical determinations by the method of Brown *et al.* (17) when urine specimens from oophorectomized-adrenalectomized or hypophysectomized patients were analyzed. It seems therefore justifiable to assume that the method em-

TABLE 3. SENSITIVITY OF KOBER METHODS IN TERMS OF THE LOWEST ESTROGEN LEVELS (μG./24 HRS.), WHICH ARE DISTINGUISHABLE FROM ZERO

Author	Estrone	Estradiol-17β	Estriol
Brown *et al.* (23)	0.64	0.60	0.84
Bauld *et al.* (29)	(0.40)*	0.75	1.00
Present study ("normal" subjects)	0.64	1.04	1.06
Present study (breast cancer cases)	1.13†	0.67†	1.60†

* Approximate value based only on 4 estimates.

† Calculated as μg./96 hrs.

ployed in the present study gave reliable estimates in the majority of breast cancer patients studied.

As a further check on specificity, in a few cases attempts were also made to extract the final Kober-color with chloroform containing 2 per cent (by weight) of p-nitrophenol and 1 per cent (by volume) of absolute ethanol, according to Ittrich (34). This treatment resulted in a shift of the absorption maximum to 538.5 mμ and considerable additional purification of the extracts. Unfortunately, this promising technique could be used only in a very few recent cases.

RESULTS

The results of the urinary estrogen assays are summarized in Table 4. From these data it appears that there are large individual variations in estrogen excretion. This is best illustrated by the fact that total estrogen excretion (*i.e.*, the sum of estrone, estradiol-17β and estriol) in the different patients varied from 84 to 471 μg. As expected, the patients excreted significantly more estrogen in the second part of the cycle (luteal phase) than in the first part (follicular phase). Ovarian irradiation resulted in a marked

TABLE 4. URINARY EXCRETION (μG./96 HOURS) OF ESTRONE (E_1), ESTRADIOL-17β (E_2), AND ESTRIOL (E_3) IN 17 PREMENOPAUSAL WOMEN

F = follicular phase; L = luteal phase; X_1 = specimens collected two months following ovarian irradiation; X_2 = specimens collected four months following ovarian irradiation; S = specimens collected two months following surgical castration.

Patient No	F			L			X_1			X_2			S			Total		
	E_1	E_2	E_3	E_1	E_2	E_3	E_1	E_2	E_3	E_1	E_2	E_3	E_1	E_2	E_3	E_1	E_2	E_3
1	15.4	3.0	64.9	11.8	4.0	87.4	14.4	7.6	5.2	8.7	1.6	1.3	4.4	0.0	4.7	54.7	16.2	163.5
2	36.0	19.5	20.3	81.1	45.8	43.4	22.7	43.8	9.7	14.9	5.4	10.9	21.3	30.0	12.4	176.0	144.5	96.7
3	5.5	0.0	7.2	38.5	1.2	46.5	11.8	3.4	4.7	13.4	7.5	6.5	8.0	0.0	0.0	77.2	12.1	64.9
4	15.0	10.8	16.2	30.6	27.7	82.0	10.2	0.0	6.4	11.5	8.4	0.0	14.1	5.7	5.2	81.4	52.6	109.8
5	23.9	0.0	6.4	38.3	0.0	26.2	31.2	13.4	9.6	41.6	37.6	6.5	7.1	0.0	7.2	142.1	51.0	55.9
6	23.0	18.3	21.0	10.6	5.6	22.4	7.1	0.0	6.8	—	—	—	8.6	6.0	0.0	49.3*	29.9*	50.2*
7	8.3	0.0	11.5	29.0	2.2	11.7	1.7	0.0	3.7	5.6	0.0	0.0	6.6	0.0	4.4	51.2	2.2	31.3
8	20.6	16.4	18.7	27.3	19.9	144.1	17.9	8.0	5.6	10.0	0.0	5.7	12.5	8.6	7.4	88.3	52.3	181.5
9	27.8	19.6	53.7	61.0	25.5	89.3	5.6	0.0	7.2	28.6	58.8	18.8	7.5	16.9	11.4	130.5	120.8	180.4
10	8.1	3.0	9.1	47.0	26.6	57.1	17.6	0.0	0.4	9.0	0.0	0.0	11.4	0.0	0.0	93.1	29.6	66.6
11	12.6	4.3	30.1	29.8	12.8	12.1	5.2	0.0	2.8	5.3	10.1	3.2	14.8	8.4	6.6	67.7	35.6	54.8
12	11.8	10.8	23.2	47.0	26.6	57.5	19.9	32.6	10.6	26.6	39.0	1.6	11.1	20.5	14.7	116.4	129.5	107.6
13	11.1	7.8	31.3	32.1	15.1	97.9	16.2	7.5	8.3	23.3	16.3	0.0	18.1	11.3	9.7	100.8	58.0	147.2
14	31.2	13.2	39.2	21.0	11.0	28.5	2.6	3.8	2.5	9.4	6.0	5.2	4.7	0.0	6.0	68.9	34.0	81.4
15	11.2	7.0	17.6	12.9	9.1	52.2	13.7	0.0	8.1	6.5	0.0	1.7	7.4	0.0	4.8	51.7	16.1	84.4
16	19.0	34.3	28.2	33.6	30.8	24.2	28.5	50.1	26.1	28.0	49.2	25.6	25.4	44.2	24.2	134.5	208.6	128.3
17	36.7	14.7	25.4	42.2	34.7	124.9	14.5	39.2	7.8	13.8	41.4	0.0	11.5	10.7	4.8	118.7	140.7	162.9
Total	*317.2*	*182.7*	*424.0*	*593.8*	*293.6*	*1607.4*	*240.8*	*209.4*	*125.5*	*256.2*†	*283.3*†	*87.0*†	*194.5*	*162.3*	*123.5*	*1602.5*	*1134.3*	*1767.4*

* Only 4 analyses.
† Only 16 analyses.

decrease in estrogen excretion. This drop was most marked in the estriol fraction; whereas the excretion of estrone or that of estradiol-17β was reduced by 40 to 50 per cent, the corresponding drop in estriol excretion was approximately 90 per cent. This discrepancy was also reflected in the mean estriol/(estrone+estradiol) ratios. These ratios were calculated for the different phases of the study as follows:

Phase of study	Mean estriol/(estrone+estradiol) ratio
follicular phase (F)	0.85
luteal phase (L)	1.13
2 months following ovarian irradiation (X_1)	0.28
4 months following ovarian irradiation (X_2)	0.16
2 months following bilateral oophorectomy (S)	0.35

Thus—provided that the method measured specifically the expected estrogens also in these breast cancer patients—it appears that ovarian irradiation particularly interferes with the process of formation and/or elimination of estriol.

From the totals shown at the bottom of Table 4 the average *daily* excretion (μg. per 24 hours) can also be calculated. Since such data facilitate a comparison with those of other workers, they are summarized in Table 5.

Statistical analysis. Due to the completely balanced design of the study, it is possible to eliminate the inter-patient variation and to assess adequately the effect of the various phases of treatment. In order to simplify the calculations, they were carried out on total estrogen values (the word "total" being used to denote the sum of estrone, estradiol-17β and estriol). Due to the great individual variations it was considered more satisfactory to assume a lognormal rather than a normal distribution of indi-

TABLE 5. AVERAGE DAILY EXCRETION OF ESTRONE, ESTRADIOL-17β AND ESTRIOL IN 17 PREMENOPAUSAL WOMEN AT DIFFERENT PHASES OF THE TREATMENT. FOR FURTHER PARTICULARS CONSULT TABLE 4

Phase of study	Estrogen excretion (μg./24 hrs.)			Total (estrone +estradiol +estriol)	Total*
	Estrone	Estradiol-17β	Estriol		
F	4.7	2.7	6.2	13.6	11.8
L	8.7	4.4	14.8	27.9	25.5
X_1	3.5	3.0	1.8	8.3	6.1
X_2	4.0	4.4	1.4	9.8	6.4
S	2.9	2.4	1.8	7.1	5.5

* Lognormal distribution of individual values assumed, *i.e.*, this value is calculated from the geometric, rather than the arithmetic means (see also Table 6).

† Only 16 analyses.

vidual estrogen values (see Gaddum, (35)). In this way the actual measurements are converted into a form which may be expected to have the best possible chance of being normally distributed. Hence, before carrying out an analysis of variance, the individual total estrogen values were converted into logarithms to base 10. A practical consequence of the adoption of a logarithmic transformation is that the mean of the logarithms, *i.e.*, the geometric mean of the observations, is taken as the most likely value instead of the arithmetic mean. Finally, in order to facilitate the computational work, each value was increased by 1. In other words the values for total estrogen were transformed according to the transformation $y = 1 + \log z$, where z indicates the original value of total estrogen (in μg. per 96 hours). These transformed values are shown in Table 6.

In Patient No. 6, one analysis was missing (*cf.* Table 5). In order to maintain the symmetrical design of the study, this missing value was calculated from the evidence obtained in the other 84 analyses, using standard sta-

TABLE 6. SUM OF VALUES OF ESTRONE+ESTRADIOL-17β+ESTRIOL TRANSFORMED ACCORDING TO THE TRANSFORMATION $y = 1 + \log z$, WHERE z INDICATES THE ORIGINAL VALUE OF "TOTAL" ESTROGEN (μG./96 HRS.)

Patient	F.–	L.–	X_1–	X_2–	S–	Total
1	2.921	3.014	2.435	2.064	1.959	12.393
2	2.880	3.231	2.882	2.494	2.804	14.291
3	2.104	2.936	2.299	2.438	1.903	11.680
4	2.623	3.147	2.220	2.299	2.398	12.687
5	2.481	2.810	2.734	2.933	2.155	13.113
6	2.587	2.794	2.143	(2.090)*	2.164	11.778
7	2.297	2.632	1.732	1.748	2.041	10.450
8	2.746	3.282	2.498	2.196	2.455	13.177
9	3.005	3.245	2.107	3.026	2.554	13.937
10	2.305	3.116	2.255	1.954	2.068	11.698
11	2.672	2.738	1.903	2.270	2.474	12.057
12	2.879	3.118	2.800	2.827	2.666	14.290
13	2.701	3.162	2.505	2.598	2.592	13.558
14	2.922	2.782	1.949	2.314	2.029	11.996
15	2.554	2.870	2.338	1.914	2.086	11.762
16	2.911	2.947	3.018	3.012	2.972	14.860
17	2.885	3.305	2.789	2.742	2.431	14.152
Total	45.473	51.129	40.607	40.919	39.751	217.879

* Missing entry calculated by the use of the general formula $y = rR + cC - G/(r-1)(c-1)$, where y is the missing item, r the number of rows, R the sum of values in the row with the missing value, c indicates the number of columns, C the sum of values in the column with the missing entry, and G is the grand total, *i.e.*, the sum of all values.

tistical techniques (36). The analysis of variance for the data of Table 6 is shown in Table 7.

A calculation of the variance ratios (F-values) reveals that both the differences between treatments and between patients are highly significant sources of variation ($P < 0.001$). The 4 degrees of freedom associated with the "between treatments" component can further be subdivided by the use of orthogonal contrasts (28, 36).

Among the many possible sets of orthogonal contrasts, the 3 sets in Table 8 have been chosen because they are relevant to this particular experiment. Thus the first set of 4 components provides the following information: surgical castration does not diminish significantly the excretion of estrogen as compared to excretion values obtained four months following ovarian

TABLE 7. ANALYSIS OF VARIANCE FOR THE DATA OF TABLE 5

Adjustment for mean 558.4854				F
Source of variation	Degrees of freedom	Sum of squares	Mean square	
Between treatments	4	5.3620	1.3405	21.13*
Between patients	16	4.8216	0.3014	4.75*
Residual error	63	3.9964	0.06343	
Total	83	13.4800		

* $P < 0.001$.

irradiation (contrast shown in the first row); ovarian irradiation diminishes significantly ($P < 0.01$) the excretion of estrogen as compared to that in the follicular phase (the contrast in the second row); and the increase in the luteal-phase values is by far the greatest source of variation between treatment effects (the contrast in the fourth row). The second set of coefficients indicates that the difference between follicular-phase values on the one hand and the mean of values obtained after ovarian irradiation on the other hand is highly significant ($P < 0.001$) (the contrast in the second row), and that there is probably no major difference between the first and second specimens analyzed following ovarian irradiation. The data of the third set of coefficients lead to similar conclusions: it can be seen that oophorectomy does not diminish estrogen excretion significantly beyond that caused by ovarian irradiation, and that estrogen excretion in the follicular phase significantly ($P < 0.001$) exceeds the mean of the values obtained following ovarian irradiation and subsequent oophorectomy.

It should be pointed out that essentially the same conclusions were arrived at, when the original untransformed data were submitted to the same

TABLE 8. ORTHOGONAL CONTRASTS CALCULATED FROM THE DATA OF TABLES 6 AND 7

Sets of coefficients	F	L	X_1	X_2	S	Contrast	Divisor	Mean square	F
I									
	0	0	0	+1	−1	1.168	34	0.04012	<1
	+1	0	−1	0	0	4.866	34	0.69641	10.98*
	+1	0	+1	−1	−1	5.410	68	0.43041	6.79**
	+1	−4	+1	+1	+1	37.766	340	4.19492	66.13†
								5.36186	
II									
	0	0	+1	−1	0	0.312	34	0.00286	<1
	+2	0	−1	−1	0	9.420	102	0.86996	13.72†
	+1	0	+1	+1	−3	7.746	204	0.29412	4.64‡
	+1	−4	+1	+1	+1	37.766	340	4.19492	66.13†
								5.36186	
III									
	0	0	+1	−1	0	0.312	34	0.00286	<1
	0	0	+1	+1	−2	2.024	102	0.04016	<1
	+3	0	−1	−1	−1	15.142	204	1.12392	17.72†
	+1	−4	+1	+1	+1	37.766	340	4.19492	66.13†
								5.36186	

* $P < 0.01$.
** $P < 0.05$.
† $P < 0.001$.
‡ $0.05 < P < 0.10$.

statistical analysis. Therefore the conclusions reached can not be attributed to the use of a especially favorable mathematical model in the statistical treatment of results.

Main conclusions from the statistical analysis. These can be summarized as follows: The greatest variation in urinary estrogen excretion in the patients studied occurred between the first and second part of the cycle, *i.e.*, it seemed to depend upon ovulation and corpus luteum formation. This greatly increased excretion was efficiently eliminated following ovarian irradiation. In fact, as a result of this ovarian irradiation, estrogen excretion became definitely lower than at the lowest point in the normal cycle (*i.e.*, during and immediately after menstruation). Surgical castration could not further reduce the post-irradiation excretion of estrogen. The

validity of some of these conclusions is subject to the condition that the adopted chemical method of analysis measured specifically the expected estrogens in the various samples obtained at different phases of the study.

DISCUSSION

When estrogens are estimated in low-titer urines obtained from patients with grossly pathologic conditions, the reliability of the adopted method becomes of paramount importance. Unfortunately it is not possible to obtain evidence for the reliability of a method in each individual case and therefore some degree of uncertainty will automatically be involved in every investigation of the type reported in the present paper. There are a few isolated reports indicating that in exceptional cases nonspecific Kober chromogenic material might interfere with the specificity of the method of Brown *et al.*, such as in oophorectomized-adrenalectomized breast cancer patients (37), but there is also plenty of evidence indicating the reliability of estimates by this method in cases of breast cancer (31, 33). It seems therefore justifiable to assume that, in the majority of cases at least, the method of Brown *et al.* (17) will most probably provide reliable estimates of the concentration of the 3 estrogens measured. Nevertheless, it should be realized, that in case the specificity of the estimates is not as high as we believe, some (but certainly not all) of the conclusions will be open to criticism.

The results presented in this paper indicate that ovarian irradiation significantly reduces the urinary excretion of estrone, estradiol-17β and estriol —at least temporarily—and that subsequent oophorectomy is not capable of further reducing the excretion of these estrogens. Since it has definitely been shown that ovariectomized patients excrete measurable and identifiable amounts of natural estrogens (38), it seems justifiable to conclude that the adrenals are a potential source of urinary estrogen in the oophorectomized human female.

As far as the rationale of ablative surgery in the treatment of metastatic breast cancer is concerned, it may be pointed out that although it has been demonstrated that the administration of estrogenic hormone to such patients may lead to an exacerbation of their condition, it has not been demonstrated beyond any doubt that the favorable clinical effects obtained following ovarian irradiation, oophorectomy, adrenalectomy or hypophysectomy are specifically due to the elimination of endogenous estrogen production. It should be realized that the critical estrogen tolerance (*i.e.*, the smallest amount of circulating estrogen which is capable of causing deterioration in the clinical condition of a breast cancer patient) is not known at present; nor is it known whether or not major fluctuations in the production and elimination of endogenous estrogens are more harmful in this re-

spect than a relatively low, even level of circulating estrogen. Furthermore it is known that a fairly large number of postmenopausal women with metastatic breast cancer undoubtedly benefit from the administration of huge amounts of synthetic estrogens (39). At any rate, if one accepts the view that the favorable therapeutic effect of oophorectomy depends upon the elimination of the principal source of endogenous estrogen production, then it would appear to be logical to remove the adrenals as well, since they are capable of secreting measurable amounts of estrogen in oophorectomized women. However, the problem is further complicated by the generally observed fact that the therapeutic effect of oophorectomy seems to be superior to that of ovarian irradiation. On the other hand, in the present investigation no difference in urinary estrogen excretion could be demonstrated following the surgical removal of both ovaries in breast cancer patients who had been treated previously with ovarian irradiation. In this connection it may be of interest to recall the recent findings of Plantin *et al.* (40), who found that the presence of ovaries in adrenalectomized breast cancer patients affects significantly the intermediary metabolism of exogenous cortisone. Thus it would appear, that ovariectomy might influence the steroid situation in breast cancer patients in more than one way.

It would also be of interest to attempt to correlate the therapeutic effect of the aforementioned operations to the urinary excretion of Kober chromogens. Since 11 of 17 patients in our series were alive at the time of writing this report, it has not been possible for us to study a possible correlation between estrogen excretion and survival-time.

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FAILURE OF DIETHYLSTILBESTROL TO AFFECT THYROIDAL ACCUMULATION AND RENAL CLEARANCE OF I^{131}*

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ABSTRACT

Diethylstilbestrol given in daily oral doses of 30.0 mg. to nonpregnant euthyroid subjects had no measurable effect on thyroidal I^{131} accumulation at twenty-four hours, or on the concentration of serum-precipitable I^{131} at twenty-four and seventy-two hours following administration of a tracer dose of I^{131}. Renal I^{131} clearance was unaffected by the treatment. A small increase in the volume of distribution of I^{131}, however, was observed. Changes in these parameters do not appear to account for the difference in the effects upon thyroidal economy of normal pregnancy and the administration of diethylstilbestrol to nonpregnant individuals.

THE thyroid gland of the pregnant woman exhibits functional evidences of increased hormone synthesis (1). It has been assumed, therefore, that the characteristic increase in concentration of serum-precipitable or hormonal iodine (SPI) observed during human pregnancy (2) must result, at least in part, from augmented production of hormone. Increases in the concentration of SPI equal to those observed in pregnant women have been induced in nonpregnant subjects by the administration of diethylstilbestrol (3). However, unlike the effect of pregnancy on the thyroid gland, administration of diethylstilbestrol does not appear to increase thyroidal accumulation of I^{131} (4). Among several explanations for this difference was the possibility that the diethylstilbestrol-induced increase in production of

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hormone was masked during tracer studies by fortuitous changes in the extrathyroidal metabolism of dietary iodide. Possible alterations which seemed worth exploring were the effect of diethylstilbestrol on thyroidal accumulation of I^{131}, rate of glandular turnover of I^{131} as estimated by concentration of serum-precipitable I^{131} (SPI^{131}), renal clearance of iodide, and the volume in which iodide is distributed.

METHODS

Thyroidal 24-hour uptakes of I^{131} were measured in 9 euthyroid adults (5 female and 4 male), before and during the oral administration of 30 mg. of diethylstilbestrol daily for from four to seven weeks. Following administration of tracer doses of 50 to 200 μc. of sodium I^{131}, concentrations of SPI^{131} were measured in 6 of the subjects at twenty-four and seventy-two hours, before and during estrogenic therapy. SPI^{131} analyses were performed by a modification of the method described by Freeberg, Ureles and Hertz (5). Concentrations of SPI^{131} were expressed as percentages of the administered dose per liter of serum.

Renal clearances of iodide were estimated in 9 recumbent euthyroid adults (6 males and 3 female) before and during the daily oral administration of 30 mg. of diethylstilbestrol for at least three weeks. The subjects were given approximately 100 ml. of tap water to drink at 10-minute intervals for one hour preceding the test and throughout the study period, thus inducing a sustained water diuresis of 7.0 ml. per minute or greater. A dose of 25 to 50 μc. of sodium I^{131} was administered intravenously immediately after emptying of the bladder. Urine collections were made over two successive 45-minute periods. The volumes of urine were measured, and a 3.0-ml. aliquot of each collection or a suitable dilution thereof was pipetted into counting tubes and counted in a well-type scintillation counter. After clotting, a 3.0-ml. aliquot of serum for each sample of blood was pipetted into counting tubes and the samples counted. All counts were related to suitably prepared standards. Counts in the serum were expressed as a percentage of the administered dose per liter of serum, and counts in the urine as a percentage of the administered dose per liter of urine. Values obtained in the samples of serum were plotted on semilogarithmic graph paper, and the best straight line drawn through the points. The volume of distribution of iodide (iodide space, IDS) was calculated from the intercept value of serum I^{131} concentration at time zero. The mean serum I^{131} level during each urinary collection was calculated from the relation:

$$B = \frac{B_1 - B_2}{\ln B_1 - \ln B_2}$$

where

B = mean serum concentration of I^{131},
B_1 = serum concentration of I^{131} at time 1, and
B_2 = serum concentration of I^{131} at time 2.

Renal iodide clearance was then calculated for each of the two urinary collection periods, and the average value taken as the renal I^{131} clearance for the particular test. Statistical analyses were performed by the "paired *t*" test. Because of the wide range of values obtained during these studies, similar analyses were also performed on the percentage change in paired values.

TABLE 1. EFFECT OF DIETHYLSTILBESTROL* ON THYROIDAL I^{131} ACCUMULATION AND SERUM PRECIPITABLE I^{131} LEVEL

Subject No.	Sex	Duration of treatment (wks.)	Thyroidal 24-hr. I^{131} accumulation (% dose)		
			Before treatment	During treatment	Difference (%)
1	M	6	28.0	30.2	+ 7.9
2	M	4	15.7	17.1	+ 8.9
3	F	4	43.7	50.9	+16.5
4	F	4	35.0	40.3	+15.1
5	F	4	48.7	46.1	− 5.3
6	M	5	31.1	31.8	+ 0.7
7	M	7	24.2	21.6	−10.7
8	F	6	42.1	37.2	−11.6
9	F	6	54.5	59.0	− 0.5
Mean			*36.4*	*37.1*	
Mean difference				*0.7*	*+ 2.2*
Standard error of mean difference				*1.1*	*1.7*
P				*> 0.1*	*> 0.1*

			Serum-Precipitable I^{131} (% dose per liter)					
			Before treatment		During treatment			
			24 hrs.	72 hrs.	24 hrs.	Difference (%)	72 hrs.	Difference (%)
2	M	4	0.010	0.016	0.013	+30.0	0.016	0.0
6	M	5	0.021	0.024	0.019	− 9.5	0.015	−37.5
7	M	7	0.024	0.019	0.016	−33.3	0.024	+26.3
8	F	6	0.010	0.043	0.009	−10.0	0.050	+16.3
10	F	4	0.020	0.049	0.021	+ 5.0	0.055	+12.2
11	F	4	0.024	0.045	0.016	−33.3	0.044	− 2.2
Mean			*0.018*	*0.033*	*0.016*		*0.034*	
Mean difference					*0.002*	*− 8.5*	*0.001*	*+ 2.5*
Standard error of mean difference					*0.002*	*11.7*	*0.003*	*10.2*
P					*>0.1*	*>0.1*	*>0.1*	*> 0.1*

* Dosage = 30 mg. daily by mouth.

RESULTS

Radioactive iodine uptake (*Table 1*). The 24-hour thyroidal accumulation of I^{131} in 9 euthyroid subjects ranged from 15.7 to 59.5 per cent of the administered dose (mean, 36.4 per cent). Following at least four weeks of

TABLE 2. EFFECT OF DIETHYLSTILBESTROL* ON RENAL I^{131} CLEARANCE AND IODIDE DISTRIBUTION SPACE

Subject No.	Sex	Duration of therapy (wks.)	Clearance (ml. per minute)			Iodide distribution space (liters)		
			Before treatment	During treatment	Difference (%)	Before treatment	During treatment	Difference (%)
1	M	6	29.6	27.5	− 7.1	27.17	29.76	+ 9.5
2	M	4	34.2	34.2	0.0	26.18	32.26	+12.3
4	F	4	43.7	50.9	+16.5	20.00	23.26	+16.3
5	F	4	24.7	25.8	+ 4.5	15.63	17.54	+12.2
7	M	7	31.7	31.8	+ 0.3	29.85	30.30	+ 1.5
8	F	6	48.2	45.6	− 5.4	27.03	25.64	− 5.1
9	F	6	27.5	30.2	+ 9.8	19.61	19.61	0.0
12	F	5	16.2	17.6	+ 8.6	18.52	20.62	+11.3
13	F	4	35.0	40.3	+15.1	17.39	17.86	+ 2.7
Mean			*32.3*	*33.8*		*22.38*	*24.09*	
Mean difference				*1.5*	*+ 4.7*		*1.71*	*+ 6.7*
Standard error of mean difference				*1.1*	*3.0*		*0.81*	*2.4*
P				*>0.1*	*> 0.1*		*<0.10*	*< 0.02*

* Dosage = 30 mg. daily by mouth.

treatment with diethylstilbestrol, these values ranged from 17.1 to 59.0 per cent (mean, 37.1 per cent). Paired estimates indicated that the uptake increased in 4 subjects during estrogenic therapy, decreased in 3 and remained unchanged in 2. Analysis by the "paired *t*" test revealed no significant difference between pretreatment and treatment mean values, whether expressed as absolute percentage uptake of the administered doses or as percentage change between paired values ($P > 0.1$).

Serum-precipitable I^{131} level (Table 1). The concentration of SPI^{131} in 6 euthyroid subjects at twenty-four and again at seventy-two hours following the administration of a tracer dose of I^{131} did not differ between pretreatment and estrogenic treatment periods ($P > 0.1$). Pretreatment concentrations ranged from 0.010 to 0.024 per cent of the administered dose per liter of serum (mean, 0.018 per cent per liter) at twenty-four hours, and ranged from 0.016 to 0.049 per cent per liter (mean, 0.033 per cent per liter) at seventy-two hours. Treatment values ranged from 0.009 to 0.21 per cent per liter (mean, 0.016 per cent per liter) at twenty-four hours and from 0.015 to 0.055 per cent per liter (mean, 0.034 per cent per liter) at seventy-two hours. Paired comparisons in the same subjects indicated that

the SPI^{131} concentration increased in 3, decreased in 1 and remained unchanged in 2 at seventy-two hours.

Renal iodide clearance (*Table 2*). The renal clearance of I^{131} in 9 euthyroid subjects ranged from 15.7 to 48.7 ml. per minute (mean, 32.3 ml. per minute) before initiation of therapy. Following at least four weeks of treatment with diethylstilbestrol, these values ranged from 17.6 to 45.6 ml. per minute (mean, 33.8 ml. per minute). The clearance increased in 5 subjects, decreased in 2 and remained unchanged in 2. There was no significant difference between these mean values, nor was a difference found when the percentage changes in paired values were similarly compared ($P > 0.1$).

Iodide distribution space (*Table 2*). The volume of distribution of I^{131} ranged from 15.6 to 29.9 liters (mean, 22.4 liters) before treatment to 17.5 to 32.3 liters (mean, 24.1 liters) during administration of diethylstilbestrol. Values increased in 6 subjects and were approximately unchanged in 3; mean values were not significantly different ($P > 0.02$). However, when expressed as percentage changes from pretreatment values, the increase in the mean space was significant to the ninety-eighth percentile level of confidence.

DISCUSSION

As measured by the methods employed, administration of diethylstilbestrol to nonpregnant euthyroid subjects is without effect on the thyroidal accumulation, glandular turnover, or renal excretion of I^{131}. Several possible explanations for the difference between the effects of pregnancy and of diethylstilbestrol therapy on thyroidal economy remain to be considered. Studies in laboratory animals suggest that natural estrogens increase thyroidal avidity for iodide (6). Increased endogenous corticoid elaboration is unlikely to account for the alterations in the metabolism of iodide during pregnancy, since the thyroidal effects of glucocorticoids are generally inhibitory (7). Whether the placenta elaborates thyrotropin is unknown. Thyrotropin-like activity has not been demonstrable in hypophysectomized guinea pigs (8), but apparently has been detected in human placentae (9). It should be noted that during the present studies the absolute quantities of I^{127} participating in the dynamic processes of thyroidal and renal iodide metabolism were not assessed. Thus, altered specific activity relationships cannot be excluded.

These studies fail to account for the difference between the thyroidal responses characteristic of pregnancy and the effects of diethylstilbestrol therapy in nonpregnant subjects. These differences are emphasized when projected against the similarities between the two, namely, increase in the concentration of SPI (3), and increase in thyroxine-binding activity of the circulating thyroxine-binding proteins (10). The finding of an increase in

the volume of distribution of isotopic iodide directs attention to the large increases in extracellular fluid volume characteristic of pregnancy (11). Thus, during pregnancy, unless dietary incorporation of iodide is increased commensurately or there is augmented recycling of inorganic iodide from accelerated breakdown of hormone, the plasma inorganic iodide level must be reduced, and an increase in the thyroidal clearance of iodide would be required for formation of even normal quantities of hormone. Since renal iodide clearance is proportional to the rate of glomerular filtration (12), the marked increase in renal blood flow and glomerular filtration observed during pregnancy (13) could also contribute to increased thyroidal avidity for iodide by further exaggerating the thyroid-renal competition for available circulating iodide. Losses of iodide to the conceptus, directed toward establishment of fetal thyroidal stores of hormone, occur throughout gestation and may begin before development of the fetal thyroid gland (14). These phenomena could result in increased thyroidal avidity for iodide even in the absence of the increased peripheral demands for maternal hormone which may exist during pregnancy. The present findings and a consideration of the hemodynamic alterations during pregnancy indicate need for further study of the metabolism of dietary I^{127} during gestation. Relative iodide deficiency could play a part in the genesis of goiter and the increase in thyroidal function observed in pregnant women.

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EFFECT OF THYROTROPIC HORMONE ON THE ACCUMULATION OF RADIOACTIVE IODINE IN THYROTOXICOSIS

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ABSTRACT

The effect of 5 U.S.P. units of thyrotropic hormone (TSH) on the accumulation of radioactive iodine (I^{131}) by the thyroid was studied in 12 cases of thyrotoxicosis. All the patients showed an increase in the thyroidal clearance rate after administration of TSH, the average increase being 30 per cent above the initial value. The change in accumulation of I^{131} was confirmed by a reduction in the urinary excretion of I^{131} in the 0–8 hour period. There was no significant change in the 24-hour thyroidal I^{131} uptake. In 2 subjects with normal thyroid function, TSH produced a threefold increase in the clearance rate. It is concluded that the thyrotoxic gland responds to TSH qualitatively in the same way as the normal thyroid gland. The significance of this finding in relation to the etiology of thyrotoxicosis is discussed.

IT HAS been shown that thyrotropic hormone (TSH) stimulates the release of organic iodine from the thyroid in thyrotoxicosis (1–5). Werner and associates (2), however, concluded on the basis of 24-hour uptake measurements that TSH did not increase the accumulation of radioiodine in thyrotoxicosis. Einhorn (6) found that in most cases there was a distinct increase in the initial accumulation rate of radioiodine in 14 patients with diffuse toxic goiter; the effect on the 24-hour uptake was equivocal.

The investigation reported here was designed to study the effect of TSH on the accumulation of radioiodine in thyrotoxicosis. The thyroidal clearance rate was chosen as the most suitable type of test for detecting a possible change in the rate of accumulation of radioiodine. The 24-hour thyroidal uptake was also measured.

MATERIAL AND METHODS

Patients

Twelve thyrotoxic patients with diffuse or nodular goiter, who had been admitted to the hospital for treatment with radioiodine, were investigated. Six of them had not had any previous treatment; the other 6 had been treated with carbimazole for periods of four to eight weeks, but stopped taking the drug at least a week before the tests were made.

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Methods

Thyroid function was assessed initially after an intravenous dose of 20–30 μc. of I^{131}. The technique for measuring the thyroidal clearance rate is described in detail subsequently. Thyroidal uptake was measured at one, two, three, four, six and twenty-four hours. Urinary excretion was measured for the periods 0–8 and 8–24 hours.

A single intramuscular injection of TSH (5 U.S.P. units) was given on completion of these measurements. A second dose of 100 μc. of I^{131} was given twenty-four hours after the administration of TSH and the described tests of thyroid function were repeated. Allowance for radioactivity in the thyroid and plasma as a result of the first tracer dose of I^{131} was made in calculating the results of the second set of tests.

Thyroidal clearance rate. The test is a modification of the method originally described by Myant *et al.* (7). *In vivo* measurements of thyroidal uptake are made with an Ekco N.5095 scintillation counter fitted with a wide angle (90°) collimator and kept at a distance of 20 cm. from the anterior surface of the neck by means of a spacing bar. Radioactivity in the neck is estimated by a counting technique based on the method described by Goodwin *et al.* (8). Alternate shielded and unshielded counts are carried out using an 11.5×8.3 cm. lead shield of 2.3 cm. thickness. The radioactivity in the shielded volume of tissue is proportional to the difference between the counts and includes both thyroidal and extrathyroidal radioactivity. In order to correct for extrathyroidal radioactivity, similar measurements are made over the thigh at a point about 10 cm. above the knee. The method of correction for extrathyroidal radioactivity is described in detail elsewhere (9).

Measurements over the neck and thigh are made for a period of thirty minutes after injection of the dose. A blood sample is taken at twenty minutes and the radioactivity in the plasma counted in a well-type liquid scintillation counter. The thyroidal clearance rate is obtained from the expression:

$$\text{Thyroidal clearance} = \frac{\text{Rate of change of thyroidal } I^{131}}{\text{Plasma concentration of } I^{131}}.$$

RESULTS

Values for the thyroidal clearance rate, the 0–8 hour urinary excretion of I^{131} and the 24-hour thyroidal uptake before and after TSH are shown in Table 1.

In every case the thyroidal clearance rate was increased after the administration of TSH. The amount by which it was increased varied considerably in the 12 patients studied and apparently did not bear any relation to the initial value (Fig. 1). In order to obtain some idea of the effect of TSH on the clearance rate in nonthyrotoxic subjects, 2 patients with normal thyroid function were studied. The clearance rate was increased from 54 to 179 ml. and from 12 to 40 ml. per minute respectively after a single injection of TSH (5 U.S.P. units).

In all patients the amount of I^{131} excreted in the 0–8 hour period was less after administration of TSH. There was no significant difference in the 8–24 hour period.

After TSH most patients showed an obvious increase in thyroidal I^{131}

TABLE 1. VALUES FOR THYROIDAL CLEARANCE RATE, 0–8 HOUR URINARY EXCRETION OF I^{131}, AND 24-HOUR THYROIDAL UPTAKE OF I^{131} BEFORE AND AFTER 5 U.S.P. UNITS OF THYROTROPIC HORMONE

Patient	Thyroidal clearance rate (ml. plasma/min.)		Urinary excretion of I^{131}, 0–8 hours (% of dose)		Thyroidal 24-hour uptake of I^{131} (% of dose)	
	Before TSH	After TSH	Before TSH	After TSH	Before TSH	After TSH
1. A.O.*	129	170	25.2	22.0	70	73
2. L.J.	142	204	22.0	19.5	45	52
3. E.K.	187	204	16.2	15.4	60	60
4. A.W.*	194	233	12.5	7.8	70	82
5. G.S.*	226	445	15.5	7.6	87	88
6. J.M.	228	367	17.0	11.0	78	86
7. A.H.	237	292	13.2	8.9	83	88
8. H.L.*	302	400	12.0	9.2	74	73
9. S.W.*	338	445	11.4	9.4	86	81
10. M.H.*	343	514	12.8	12.3	73	74
11. S.G.	530	604	6.0	2.4	66	67
12. S.F.	795	960	7.2	5.2	76	75

* Patients previously treated with carbimazole.

uptake at one, two, four and six hours. At twenty-four hours this difference was no longer apparent.

The patients who had been previously treated with carbimazole did not appear to differ from the untreated patients in their response to TSH.

DISCUSSION

The changes in the thyroidal clearance rate and in the urinary excretion of I^{131} confirm the observation that TSH stimulates the accumulation of iodine by the thyroid in thyrotoxicosis. It was not surprising to find that there was no significant difference in the 24-hour uptake values. In thyrotoxicosis the thyroidal clearance of iodide is much greater than the renal clearance of iodide and under these conditions a further increase in the thyroidal clearance rate has a relatively slight effect on the proportion of the dose accumulated by the thyroid at twenty-four hours. A slight increase in the total amount of I^{131} concentrated by the thyroid might well be offset by an increase in the rate of discharge of labelled hormone from the gland. The 24-hour uptake may be satisfactory for studying the effects of thyrotropic stimulation in subjects with normal thyroid function, but it is not suitable for similar studies in hyperthyroidism.

It may be assumed, since TSH stimulates both iodine uptake and release

of hormone in thyrotoxicosis, that the intermediate stages of hormone synthesis are similarly affected. The thyrotoxic gland therefore responds to TSH qualitatively in the same way as the normal thyroid gland.

The change in the thyroidal clearance rate in the 12 thyrotoxic patients was extremely variable, the average increase being 30 per cent above the initial value. In the 2 nonthyrotoxic subjects there was in each case a threefold increase in the clearance rate after administration of TSH. It seems

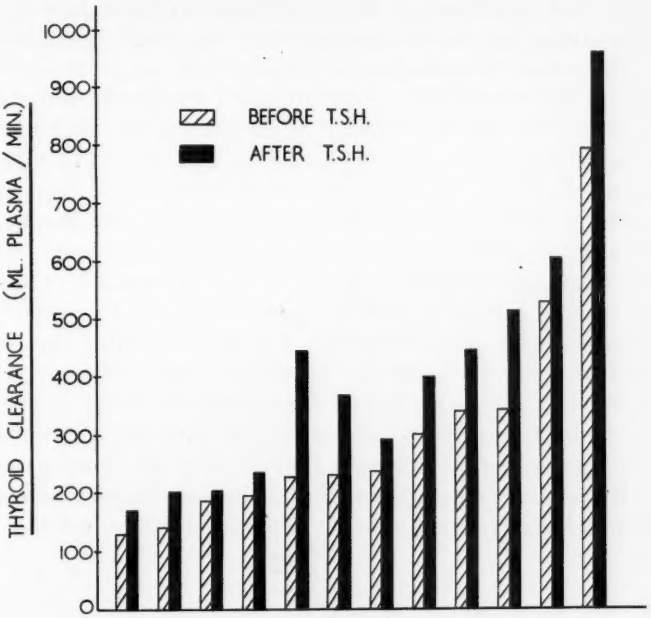

FIG. 1. Values for thyroidal clearance rate before and after 5 U.S.P. units of TSH in 12 patients with thyrotoxicosis.

likely, so far as the effect of TSH on the accumulation of iodine is concerned, that there is a quantitative difference between normal and thyrotoxic subjects. Such a difference was found when the effect of TSH on hormone release was investigated (4).

The dose of TSH used in this investigation was approximately three times the amount required to produce a maximal effect on thyroidal uptake in normal subjects (6). It is quite likely that the dose response differs in thyrotoxicosis and that larger doses of TSH might have had a greater effect on the clearance rate. There must, however, be a limit for the iodide extraction ratio in hyperplastic thyroid glands, and with clearance rates of the

order of 1000 ml. per minute, extraction is probably almost completely efficient (10). In these circumstances it is unlikely that a larger dose of TSH would have much effect on the clearance rate.

It has been shown by other workers that in thyrotoxicosis the uptake of I^{131} by the thyroid is not suppressed by the administration of thyroid substance (2, 11–13), and that TSH stimulates discharge of hormone from the gland. The behavior of the thyroid in response to stimulation or suppression has been analyzed in relation to the etiology of thyrotoxicosis. Werner *et al.* (13), relying largely on the fact that triiodothyronine fails to suppress thyroidal uptake in thyrotoxicosis, concluded that the disease is unlikely to be due to thyrotropic stimulation. Greer and Shull (4), on the other hand, found that thyrotoxic patients required about five or six times as much TSH to increase the rate of secretion of hormone as did normal patients, and postulated that exogenous TSH would not be expected to influence the thyroidal secretion rate until endogenous production had been exceeded. They concluded that their findings were compatible with the hypothesis that Graves' disease is due to increased secretion of TSH by the pituitary.

It seems clear, since thyroid function is not suppressed by the administration of thyroid hormone, that the pituitary-thyroid relationship does not function normally in thyrotoxicosis. The data presented here show that the thyroid responds normally to thyrotropic stimulation in thyrotoxic patients, although the magnitude of the response is probably less than in normal subjects. Hence the breakdown in the pituitary-thyroid relationship can be ascribed to the failure of thyroid hormone to suppress thyroid function. It remains a possibility that the pituitary becomes overactive in thyrotoxicosis because it is no longer suppressed by normal thyroid hormone.

Acknowledgment

I wish to thank Mr. F. G. Mason, M.S.R., for technical assistance.

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FURTHER STUDIES OF THE KINETICS OF GLUCOSE UTILIZATION.* I. A NEW METHOD OF DATA ANALYSIS

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ABSTRACT

Evidence is presented to show that the extreme variation in rate constants for blood glucose disappearance reported by different laboratories is due to the use of an incorrect equation and/or an incorrect method of data analysis. It is demonstrated that graphic (semi-log) methods are too insensitive to be used to justify equations describing blood glucose disappearance. The correct equation is shown to be one of exponential form containing an added constant term which in the normal subject is significantly less than the fasting level of blood glucose. A new method of data analysis (slope analysis) is described which results in the same specific rate constant, K, regardless of the equation employed. This method is made practical by the introduction of a mechanical slope reader which eliminates excessive calculations.

FOR many years attempts have been made to replace oral glucose tolerance tests with intravenous techniques so as to eliminate the variable of intestinal absorption and to obtain a more quantitative index of carbohydrate metabolism (1–13). Intravenous methods permit the calculation of a specific rate constant for glucose utilization (K) from a relationship of blood glucose and time.[1]

Ordinarily, the application of mathematical principles to a biologic system results in a better understanding of the system. However, in this instance it has led to confusion and disagreement. Table 1 illustrates the extremely wide variation in mean K values (.0134 to .0519) reported in normal subjects, even though only 2 experimental techniques—single rapid injection and continuous infusion of glucose—have been used. No explanation has been offered for the large variation in reported mean K values (.0134 to .0420) resulting from the single-injection technique, nor for the difference in K values obtained with the 2 techniques.

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* Glucose utilization is defined here as the disappearance of blood glucose.

[1] The specific rate constant, K, is a proportionality constant defined as the rate of glucose utilization when the blood glucose concentration is equal to unity.

TABLE 1. SPECIFIC RATE CONSTANTS FOR GLUCOSE UTILIZATION

Investigators & Reference	No. of subjects	Method*	Equation No.	Mean K†	Standard Error	Range
Duncan (1)	20	S.I.	I	.0137	.0022	.0097–.0189
	20	S.I.	II	.0368	.0040	.0315–.0462
Ikkos & Luft (2)	50	S.I.	I	.0144		
(3)	37	S.I.	I	.0172	.0014	
Amatuzio *et al.* (4)	70	S.I.	II	.0370	.0054	.0297–.0485
	70	S.I.	I	.0134‡	.0027	.0098–.0182
Lozner *et al.* (5)	60	S.I.	I	.0196‡	.0030	
	60	S.I.	II	.0357‡	.0037	
Bastenie *et al.* (6)	20	S.I.	I	.0155	.0031	
	18	S.I.	I	.0184	.0068	
Marks & Bishop (7)	19	S.I.	I	.0197	.0062	.0131–.0347
	19	S.I.	II	.0420	.0152	.0278–.0796
Silverstone *et al.* (8)	35	S.I.	I	.0137	.0065	.0067–.0341
Greville (9)	34	S.I.	III	.0270		.0160–.0390
Holten *et al.* (10)	22	S.I.	I	.0177	.0054	
Hlad *et al.* (11)	26	S.I.	III	.0308	.0120	.0155–.0610
	69	C.I.	IV	.0400	.0126	.0196–.0825
Jokipii & Turpeinen (12)	24	C.I.	IV	.0519		.0400–.0760

* S.I. = single injection; C.I. = continuous infusion.
† K is given in units of reciprocal time (min.$^{-1}$).
‡ Analysis by Duncan (1).

Hamilton and Stein (13) introduced a simple exponential equation[2] to describe blood glucose disappearance:

$$C_g = C_0 e^{-Kt} \qquad \text{(I)}$$

[2] Definition of symbols:
C_g—Blood glucose concentration at any time (t).
C_f—Fasting level of blood glucose.
C_0—Theoretical maximum blood glucose level at the time when mixing is complete ($t = 0$).
K—Specific rate constant for glucose utilization.
C_{eq}, C_{eq}'—Theoretical blood glucose concentrations at equilibrium ($t = \infty$).

Although they reported blood glucose data which formed obvious curves when they were plotted against time on semi-logarithmic paper, they applied their equation to small arbitrarily selected portions of the curves, which they treated as straight lines. Many others (2, 3, 6, 10) have used this equation, applying it to either the initial or final segments of a blood glucose disappearance curve which obviously did not satisfy the equation in its entirety.

Amatuzio *et al.* (4) assumed a first-order reaction to describe blood glucose disappearance and proposed the following equation:[2]

$$C_g = (C_0 - C_f)e^{-Kt} + C_f \qquad \text{(II)}$$

The introduction of a fasting blood glucose concentration term (C_f) into the simple exponential equation resulted in an equation which, some have claimed, describe the data better (1, 7, 8). Others have claimed that this equation is applicable only to the initial portion of the curve (2).

Many years prior to this, Greville (9) proposed a similar equation. However, he did not define the constant term of his equation as the fasting level, because experimentally it was well below that level in normal subjects. He attributed no physiologic significance to this term. Greville's equation described the blood glucose curve for his entire experimental period (eighty-five minutes). Jokipii and Turpeinen (12) first employed a continuous infusion method. They derived an equation also based upon the assumption of a first-order reaction for glucose disappearance. Mathematically it was similar to equation II, except for a constant infusion-rate term. However, in only half of their experiments could their data be described by smooth curves.

The foregoing equations were either arrived at empirically, or were derived on the assumption that blood glucose disappears according to a first-order chemical reaction. In a previous study we showed that it was not necessary to assume a first-order reaction (11). Instead, experimentally established relationships between blood glucose concentration and peripheral and hepatic glucose utilization reported by others (14–18) were used to derive equations which more accurately described blood glucose disappearance for extended experimental periods. These equations[2] for the two different techniques of glucose injection are:

Single injection:

$$C_g = (C_0 - C_{eq})e^{-Kt} + C_{eq} \qquad \text{(III)}$$

Continuous infusion:

$$C_g = (C_f - C_{eq}')e^{-Kt} + C_{eq}' \qquad \text{(IV)}$$

Each of the foregoing investigators considered his equation correct because a plot of some function of blood glucose concentration *vs.* time on semi-logarithmic paper resulted in a straight line.

It is the purpose of this paper: (a) to demonstrate that the widespread disagreement in K values is due to the use of incorrect equations, usually justified by insensitive methods of data analysis; (b) to present experimental evidence showing which of the proposed equations most precisely describes blood glucose disappearance; and (c) to describe an improved method of data analysis which is practical and which yields K values independent of the equations used to describe blood glucose disappearance.

METHODS

Subjects. The subjects employed in this study were men between the ages of 23 and 71 with no known defects in carbohydrate metabolism. They were hospitalized for uncomplicated, non-metabolic illnesses. Each man received at least 250 Gm. of dietary carbohydrate daily for three days before the study, and fasted for fourteen hours immediately preceding it. All subjects remained in bed in a special room throughout the test. The tests were begun at 8 A.M., after the subject had been at rest in a test bed for sixty to ninety minutes. Fifty-two studies were carried out on 34 pateints, but 2 studies (on 1 patient) had to be omitted because the data could not be described by a smooth curve. When 2 different tests were made in the same subject, they were performed one week apart. The total study was carried out over a period of one year.

Procedures. In the single-injection studies, a dose of 25 Gm. of glucose in approximately 200 ml. of distilled water was injected intravenously over a period of 4 ± 2 minutes. The continuous infusion (intravenous) studies were performed using a 10 per cent glucose solution and a Bowman constant infusion pump. Infusion rates were constant in the same subject, but varied in different subjects from 287 to 372 mg. of glucose per minute. Capillary blood (finger), which has the same glucose concentration as arterial blood (14), was obtained at intervals of five to ten minutes for a period of sixty minutes. Samples were analyzed for glucose by the Somogyi-Nelson method (19). Care was taken to minimize glycolysis by the addition of NaF and immediate refrigeration. Urine was collected at the end of each test and analyzed for total reducing substance by the quantitative Benedict method (20).

Data analysis. Blood glucose data from the foregoing studies were initially plotted on large linear graph paper ($11 \times 16\frac{1}{2}$ inches, ruled in millimeters), representing blood glucose concentration (C_g) as a function of time (t). The best smooth curve through the experimental points was then drawn. For 100 points in 10 randomly selected tests, the average difference from the actual point to the corresponding point on the smooth curve was $1.0 \pm$ a standard deviation of 0.8 per cent.

Two methods were used to obtain the specific rate constant (K) from the smooth curve. The first, a graphic method previously described by us (11), involved the use of either equation III or IV (see Introduction) and the estimation of C_{eq} or $C_{eq'}$ from the curve by extrapolation. The difference between these equilibrium values and the actual blood glucose concentration was then plotted as a function of time on semi-logarithmic paper. The slope of the resulting straight line is K. Minor adjustments of the estimated equilibrium values were frequently necessary in order to obtain a good straight line through the points of the semi-logarithmic plot.

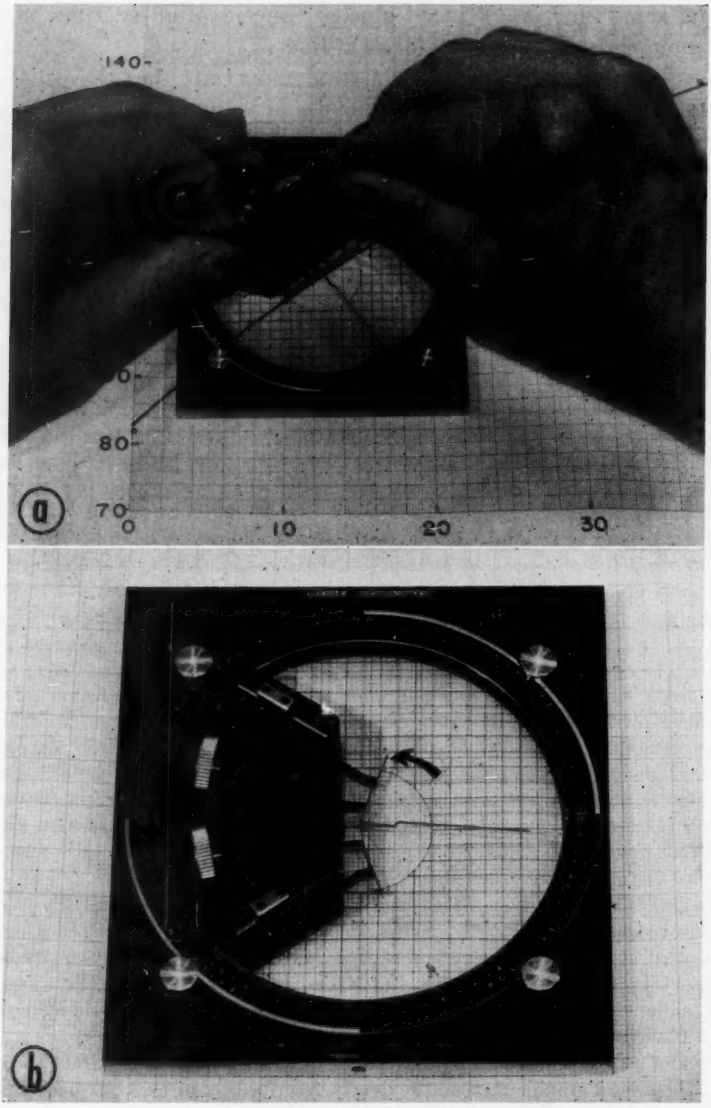

FIG. 1. a. The Derivimeter® in use for reading the slope of a curve. b. Details of the Derivimeter® showing the flexible steel spring (arrow) which may be manually adjusted to fit the contour of any curve.

The second method involved the measurement of the slope of the blood glucose-time curve at multiple points (Slope Analysis). Traditional mathematical methods for measuring a slope at a point on a curve are difficult and very time-consuming. These disadvantages were circumvented by the use of the mechanical slope reader[3] illustrated in Figure 1. The slope (dC_g/dt) of the blood glucose curve was measured at five-minute intervals and replotted as a function of blood glucose concentration on linear paper. In the latter graph a straight line is obtained, the slope of which is the specific rate constant, K.

RESULTS

Some characteristics of exponential functions

There are several methods (21) for finding empirical equations to represent experimental data. When the empirical equation is assumed to be of simple exponential form, a semi-logarithmic plot of the data is the most convenient method of testing the suitability of the equation. However, the logarithmic form is an insensitive way of showing change.[4] This method is adequate only when extremely precise measurements can be made and when the equation very accurately describes the behavior of the system. If these conditions are not satisfied, data representing a true curve may be fitted erroneously to a "good" straight line (see subsequent discussion).

It is pertinent to examine other characteristics of exponential functions. The left side of Figure 2 illustrates a simple exponential function (line F) plotted on semi-logarithmic paper according to equation 1. This equation was deliberately altered by adding a constant (C) to the right side, yielding equation 2. The new function is represented by curve F'. Mathematically, line F must be a straight line whereas line F' must be a curve. Nevertheless, the best straight line through the points of the curve F' was obtained by the method of least squares. The difference between the line and the curve was calculated for points at ten-minute intervals (Table 2). It should be noted that when the equation is changed by the addition of a constant, the K value associated with the best straight line through the points of the new curve is considerably decreased (48 per cent). This occurs despite the fact that any point on the best straight line does not deviate appreciably (mean deviation = 4.3 per cent) from corresponding points on the curve. In the graph on the right side of Figure 2, the curve (F') was arbitrarily divided in half ($t = 35$) and the best straight lines through each of the 2 segments

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[3] Derivimeter®, available from Gerber Scientific Instrument Co., Hartford, Conn This device is easy to use and requires no special skill. The accuracy of the instrument was tested by allowing unskilled personnel to measure known slopes of an exponential curve. The average error of 36 measurements by 3 observers was ±2 per cent.

[4] For example, during an intravenous glucose tolerance test, the blood sugar level may fall from 300 to 100 mg. per 100 ml., a change of 67 per cent. If these same values are expressed as logarithms, the change in the logarithms is only 19 per cent.

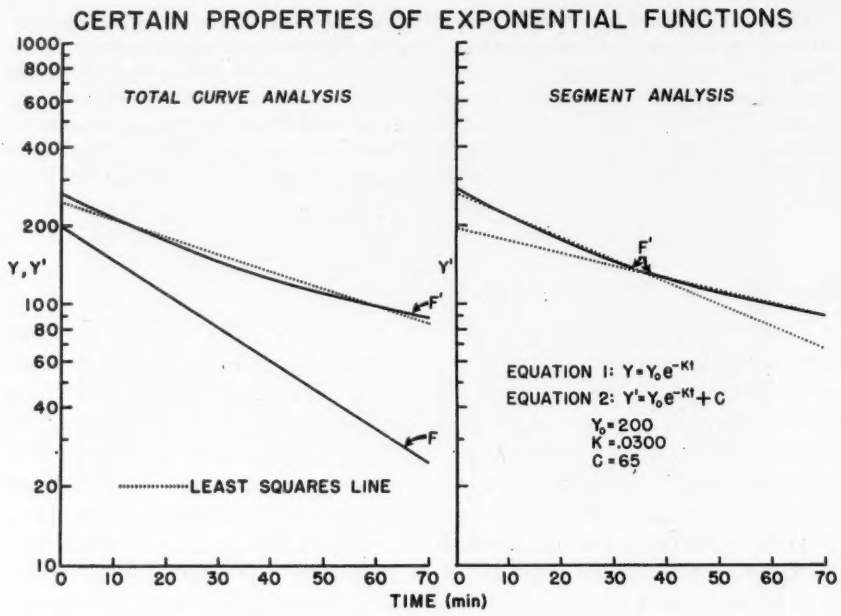

FIG. 2. Line F is plotted according to equation 1. Line F' in both graphs is plotted according to equation 2; however, in the graph on the right, it has been divided into two segments. The dotted lines represent the best straight lines (least squares method) through the data of F'.

TABLE 2. CERTAIN PROPERTIES OF EXPONENTIAL FUNCTIONS

Time (t)	Y'	Best straight-line values (total curve)	% Deviation	Best straight-line values (segment)	% Deviation	Best straight-line values (segment)	% Deviation
0	265	246	7.2	262	1.1		
10	213	211	0.9	215	0.9		
20	175	180	2.9	176	0.5		
30	146	154	5.5	144	1.3		
40	125	132	5.6			123	1.6
50	109	113	3.7			110	0.9
60	98	96	2.0			98	0.0
70	89	83	6.7			88	1.1
Mean			4.3		1.0		0.9
K	.0300	.0156		.0199		.0113	

were obtained. In this example, the best straight lines deviate even less from the corresponding curves. The magnitude of deviation is obviously dependent upon the length of the segment. For example, when segments representing half of the data were analyzed as described, the mean error did not exceed 1.0 per cent (Table 2). Analysis of segments of the curve yields an improved fit of the data to a straight line, yet the resulting K value may deviate even further from the true value, dependent upon which segment of the curve is chosen for analysis. It should be pointed out that the quality of fit of the best straight line to the data of the curve is influenced not only by C and t, but also by K and Y_0 (unpublished data).

The curve F' simulates a blood glucose disappearance curve following a single injection of glucose. However, technical errors and physiologic variations reduce the probability of observing such ideal data. Although it would be difficult to determine the total error in this process, a 5 to 10 per cent error would not be unreasonable. All the differences between the curves and the best straight lines (Table 2) are less than this estimated error.

Theory of slope analysis method

Since there might be a theoretical basis for the marked differences in K values which result from the various equations describing blood glucose disappearance, it is important to consider the theoretical slopes of the proposed equations. Mathematically, the slope is expressed as the first derivative of blood glucose concentration with respect to time. The first derivatives of equations I–IV (see Introduction) in linear form are:

Single injection

$$\frac{dC_g}{dt} = -KC_0e^{-Kt} = -KC_g \qquad \text{(IA)}$$

$$\frac{dC_g}{dt} = -K(C_0 - C_f)e^{-Kt} = -KC_g + KC_f \qquad \text{(IIA)}$$

$$\frac{dC_g}{dt} = -K(C_0 - C_{eq})e^{-Kt} = -KC_g + KC_{eq} \qquad \text{(IIIA)}$$

Continuous infusion

$$\frac{dC_g}{dt} = -K(C_f - C_{eq}')e^{-Kt} = -KC_g + KC_{eq}' \qquad \text{(IVA)}$$

In the single-injection system, if blood glucose (C_g) is plotted as a function of time on linear paper, the slope (dC_g/dt) of the curve at any point (C_g) may be obtained. If this slope is then plotted against the corresponding blood sugar concentration on linear paper, equations IA, IIA and IIIA

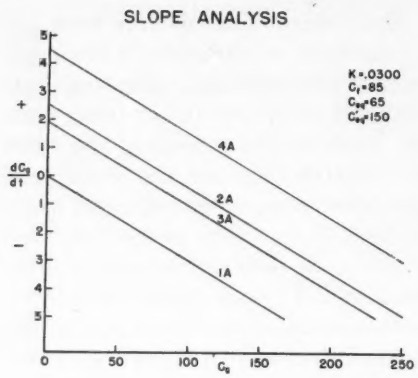

FIG. 3. The slope (dC_g/dt) of a typical blood glucose-time curve is plotted as a function of blood glucose concentration (C_g) for each of the single injection equations (IA—IIIA) given in the text. Note the identical slopes and the variation in the intercepts. For clarity, the continuous infusion equation (IVA) is shown, using the same K value.

TABLE 3. COMPARISON OF SPECIFIC RATE CONSTANTS* MEASURED BY TWO METHODS

Subject	Age (yrs.)	Single injection		Continuous infusion	
		K_e	K_d	K_e	K_d
L.H.	60	.0288	.0270	.0282	.0278
R.T.	69	.0288	.0352	.0417	.0397
J.F.	71	.0205	.0196	.0303	.0293
C.B.	56	.0190	.0180	.0338	.0334
C.H.	54	.0223	.0209	.0438	.0473
J.L.	70	.0246	.0196	.0353	.0344
A.S.	62	.0200	.0184	.0332	.0341
E.S.	58	.0184	.0172	.0362	.0341
N.M.	62	.0363	.0361	.0583	.0480
C.P.	54	.0403	.0383	.0285	.0342
L.B.	63	.0194	.0181	.0333	.0336
R.B.	49	.0290	.0329	.0336	.0334
C.M.	60	.0310	.0333	.0392	.0340
D.F.	61	.0208	.0214	.0303	.0276
W.M.	62	.0432	.0439	.0265	.0292
N.M.	57	.0460	.0446	.0360	.0346
L.C.	66	.0250	.0242	.0397	.0402
Mean		.0278	.0276	.0358	.0350
Standard error		.0089	.0095	.0076	.0059

* K_e = The specific rate constant obtained by using an extrapolated equilibrium value and a semi-logarithmic plot of increment data (Fig. 4B and E).

K_d = The specific rate constant obtained by the slope analysis technique described in the text (Fig. 4C and F).

K values given in units of reciprocal time (min.$^{-1}$).

demand that a linear relationship be obtained with a slope of K. This is illustrated in Figure 3. *Thus, for the same data, it is apparent that all single-injection equations expressed in this form yield the same K value!* The difference in equations is manifested only by the difference in the vertical axis intercept, which is dependent upon the constant term. It should be noted that the form of the continuous infusion equation (IVA) is identical with that of single-injection equation IIIA. For purposes of clarity in Figure 3, equation IVA is shown using the same K value as that obtained from the single-injection equations. Theoretically, K values obtained from the 2 systems should be identical, provided the same physiologic response is elicited (11).

Application of slope analysis method

The slope analysis technique was used to measure K values from the single-injection and continuous-infusion data of 17 subjects. In addition, the same data were analyzed for K by the graphic method previously reported by the authors (11). The results are given in Table 3 and an example is shown in Figure 4. The slope analysis technique yielded a positive intercept in every case, indicating the presence of a constant term in the exponential form of the equation. With either the single-injection or the continuous-infusion technique, K values obtained by the slope analysis method did not differ significantly ($P = .40$) from those obtained by the graphic method. However, there was a significant ($P = .01$) difference between the single-injection K and the continuous-infusion K.

In 16 subjects, glucose was administered by constant infusion for sixty minutes. Blood samples were obtained during the infusion and for the following sixty minutes. In this way both appearance and disappearance curves of blood glucose were obtained in the same subject under the same conditions. K values for each curve were obtained by applying the slope analysis technique. The data from one study are illustrated in Figure 5 and the pertinent data from all studies are presented in Table 4. Two results are clearly evident: (a) The K values for the appearance and disappearance curves do not differ significantly ($P = .54$); and (b) C_{eq} (calculated from equation IIIA) is significantly ($P < .001$) less than C_f.

The urine collected at the end of each test contained a negligible amount of total reducing substance; the mean amount was 2 per cent of the administered glucose.

DISCUSSION

Critique of equations and methods of data analysis

All equations, including those of the authors, which have been used (1–13) to describe blood glucose disappearance were justified by the use of

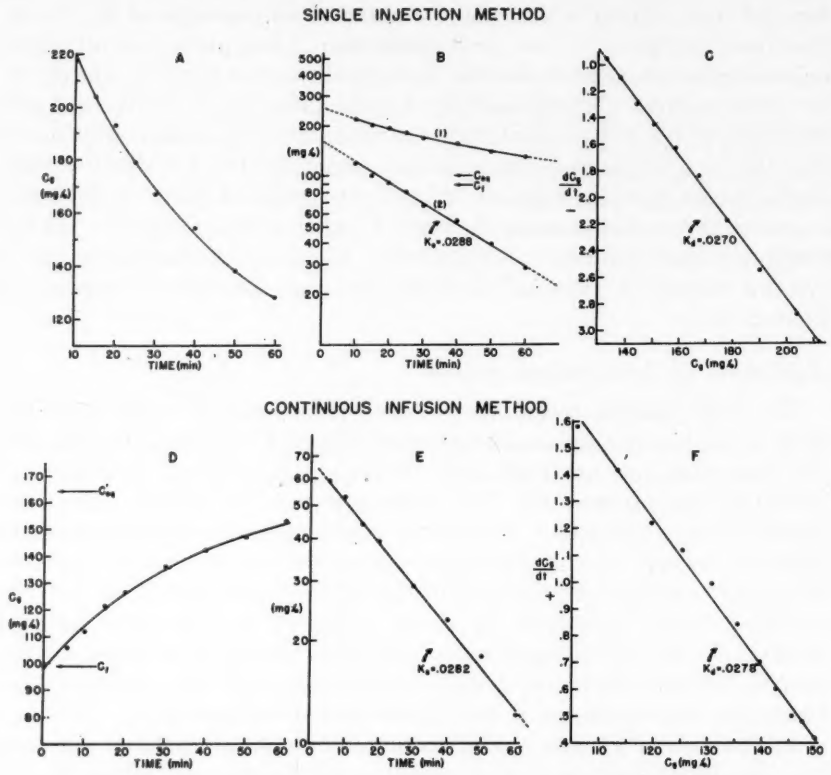

FIG. 4. Figures A and D represent linear plots of blood glucose data for both methods of glucose administration.

Figures B-2 and E represent the semi-logarithmic plots obtained after estimating the equilibrium values (C_{eq} and C_{eq}') by extrapolation. B-1 represents the original data plotted on semi-logarithmic paper. Note the diminished curvature in B-1 when compared to the curve in A.

Figures C and F are linear graphs of the slope (dC_g/dt) plotted against glucose concentration. All data were obtained on Subject L.H.

graphic and tabular suitability tests (21). Equations were considered suitable if a graph of the logarithm of a function of blood glucose concentration plotted against time appeared to be a straight line. A thesis of this paper is that the graphic procedure, although technically correct, is too insensitive to establish the validity of such equations. To support our thesis, we reviewed certain characteristics of an exponential function deliberately altered by the addition of a constant. This procedure was pertinent because all equations proposed to describe blood glucose disappearance are ex-

ponential and differ only by a constant term. It was shown that if such an equation is altered in this manner, a semi-logarithmic plot of the resulting data must be a curve. Yet the best straight line through these data does not deviate enough from the curve at any point to exceed the total error expected in procedures of this kind. If the best straight lines are determined for only portions of the curve, even better fits are obtained. The flaw of this procedure is that the specific rate constants associated with the best straight lines through all or parts of the curve not only differ from each other, but also differ markedly from that of the true function. Dependent upon the values of the constants of the equations, an infinite number of erroneous K values may be obtained from very satisfactory straight lines

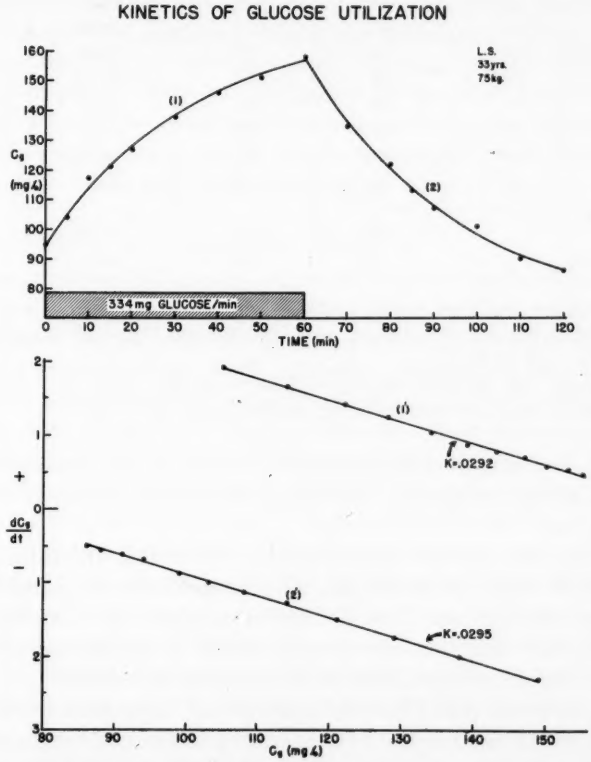

FIG. 5. The upper graph represents blood glucose concentration *vs.* time. Curve 1 is the appearance curve obtained during the continuous infusion of glucose. Curve 2 is the disappearance curve obtained immediately after the cessation of glucose infusion. The lower graph is the analysis for the specific rate constants of the upper curves according to the slope analysis technique.

TABLE 4. COMPARISON OF SPECIFIC RATE CONSTANTS, UTILIZING CONSECUTIVE APPEARANCE AND DISAPPEARANCE CURVES OF BLOOD GLUCOSE IN THE SAME SUBJECT*

Subject	Age (yrs.)	C_f	C_{eq}	K_1	K_2
A.T.	42	83	61	.0387	.0432
R.S.	38	82	68	.0302	.0336
E.F.	27	85	62	.0389	.0564
E.Q.	43	86	68	.0329	.0374
A.B.	32	100	71	.0527	.0600
R.W.	34	92	60	.0519	.0364
E.S.	37	83	68	.0374	.0329
J.H.	23	89	74	.0195	.0254
R.G.	33	87	75	.0355	.0304
L.S.	33	95	69	.0292	.0295
C.M.	36	86	47	.0164	.0193
J.K.	36	91	73	.0480	.0580
J.C.	27	92	78	.0482	.0622
F.R.	67	105	103	.0350	.0232
M.M.	35	86	53	.0286	.0251
J.G.	40	84	82	.0547	.0528
Mean		89	70	.0376	.0390
Standard Error		6.5	12.7	.0113	.0145

* C_f = Fasting blood glucose concentration (mg./100 ml.).
C_{eq} = Equilibrium blood glucose concentration from the single-injection equation (III) (mg./100 ml.).
K_1 = Specific rate constant during continuous infusion (min.$^{-1}$).
K_2 = Specific rate constant following continuous infusion (min.$^{-1}$).

through all, or portions of the data. This is one of the 2 explanations (see subsequent discussion) for the wide range of K values reported in the literature.

It should be pointed out that some of the reported equations and methods of analysis may be useful clinically to demonstrate abnormalities in carbohydrate metabolism (2, 4, 7). The results may even be highly reproducible (4). However, this does not constitute evidence that the equation employed to describe blood glucose disappearance is correct.

Greville observed that when the slope of the blood glucose curve (linear plot) was plotted against the corresponding blood glucose value, a linear relationship with a positive intercept was obtained (9). The slope of the new line is the specific rate constant (K) and the positive intercept indicates the existence of an added constant term in the exponential equation. Greville's method, although mathematically correct, has not been used,

probably because the accurate measurement of a slope at a point on a curve is difficult and time-consuming. We have shown that a mechanical slope reader can be used to eliminate the numerous calculations ordinarily involved in slope measurement. This device is simple to use and is accurate even in the hands of unskilled personnel. We have also shown from a study of the theoretical slopes of the proposed single-injection equations, that slope analysis yields a K value which is not dependent upon the equation employed. If others had analyzed their data in this manner, the mean K values for comparable groups of subjects would have been similar. The fact that this is not so (Table 1) indicates that many investigators have used an incorrect equation or an insensitive method of data analysis.

Our data show that the correct equation to describe blood glucose disappearance must be exponential and must contain an added constant term. In addition, the correct equation, with proper graphic analysis, must yield a K value not significantly different from that obtained by slope analysis. The derived equation previously proposed by the authors (11) and the empirical equation of Greville (9) both satisfy these conditions. None of the other proposed equations fulfills these requirements, and therefore none of them describes blood glucose disappearance correctly.

Effect of the method of glucose administration

It has been reported (11, 12) that K values obtained with the continuous-infusion system are significantly higher than those obtained with the single-injection system, but in these studies the 2 systems were not used in the same subject. In the present study a similar observation was made even when K values were measured by the 2 systems in the same subject (Table 3). However, when consecutive appearance and disappearance curves of blood glucose (in the same subject) were analyzed, the K values for the 2 curves were not significantly different (Table 4). In these studies, the disappearance curve simulated the curve obtained when the single-injection system was used, but the single rapid glucose load with its resultant high blood glucose levels was avoided. Blood glucose concentration was always lower than 170 mg. per 100 ml., whereas it always exceeded 200 mg. per 100 ml. when a single injection of glucose was employed. These findings indicate that when the single-injection system is employed, the specific rate constant is dependent upon the method of glucose administration or the blood glucose level. With the continuous-infusion system, the specific rate constant is independent of the rate of glucose infusion (250 to 630 mg. per minute) and of the blood glucose level (75 to 250 mg. per 100 ml.) (11). It appears that continuous infusion is the superior method because it yields the stable K value and results in a more physiologic blood glucose pattern.

Relationship between C_{eq} and C_f

The relationship between C_{eq} and C_f is of both theoretical and clinical interest. We have previously reported that with the single-injection method, C_{eq} was significantly below C_f (11). The same relationship ($C_{eq} < C_f$) was noted in the studies in which the disappearance curve of blood glucose was observed following cessation of continuous glucose infusion (Table 4). Thus both methods of glucose loading alter the state of the system. Since the K values in the latter studies did not change, it would appear that the relationship between C_{eq} and C_f is the more sensitive means of detecting alterations in the state of the system. This finding has already proved useful clinically, and may have some physiologic significance. In a series of 17 patients with mild diabetes (unpublished data), we found that C_{eq} exceeded C_f in every case (in contrast to results obtained in the normal subject), whereas K values were not significantly different from normal.

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FURTHER STUDIES ON THE RENAL ACTION OF GLUCAGON

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ABSTRACT

Studies of the action of glucagon on the renal excretion of uric acid, total nitrogen and urea nitrogen are reported. A single dose of glucagon was administered to each of 14 normal subjects and 4 patients with gout. Seven normal subjects who received glucose infusions served as controls. In addition, 2 of the patients with gout were given glucagon three times daily for several days while they were receiving a constant diet. Glucagon caused an immediate increase in uric acid excretion which persisted at least one hour. However, this increase in uric acid was not detected in 12-hour urine collections from 2 patients receiving 3 daily injections of glucagon. Evidence is presented that the immediate uricosuric effect is not secondary to the hyperglycemic action of glucagon and that it may be due to an action on the renal tubule. Administration of glucagon resulted in a significant but small increase in total nitrogen and urea nitrogen excretions. Because these changes closely paralleled changes in endogenous creatinine clearance they are considered secondary to variations in glomerular filtration rate.

IT HAS previously been reported that highly purified, crystalline glucagon increases the renal excretion of sodium, potassium, chloride, inorganic phosphorus and I^{131} in the dog (1, 2) and increases the renal clearance of sodium, potassium, chloride, inorganic phosphorus and bicarbonate in normal man (3–5). These effects were shown to be independent of the hyperglycemic action of glucagon and probably due to an effect on the renal tubule. In this paper the effect of glucagon on the renal excretion of certain nitrogenous compounds in normal subjects and patients with gout is described.

METHODS

Twenty-five hospitalized, ambulatory, male patients in good general condition were studied. Twenty-one of the group (the "normal" subjects) were convalescing from non-renal, non-metabolic illnesses; the remaining 4 had chronic gouty arthritis in remission. All received a regular hospital diet and were in good nutritional state. Eighteen patients were studied before and after a single dose of glucagon; the remaining 7 (normal controls), before and during an intravenous infusion of glucose.

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Renal clearance techniques were used. Subjects were studied in a postabsorptive state (14-hour fast), and in a special room after a rest period of sixty minutes in the test bed. Water (1 to 2 liters) was administered orally prior to, and throughout the study in order to insure adequate urine flow. Urine was collected over ten-minute periods through an indwelling, multiholed, soft rubber catheter, and each collection was concluded with multiple washouts of water and air to insure complete collections. In each study there were 3 consecutive control periods followed by 6 test periods. During the control periods isotonic sodium chloride was infused intravenously at a constant rate (2.5 to 4.8 ml. per minute) by means of a Bowman infusion pump.

Venous blood samples were collected at the midpoint of each ten-minute period in heparinized syringes through an indwelling, thin-walled needle in the antecubital vein, and the plasma was separated immediately by centrifugation. The plasma and urine samples were analyzed for uric acid (6), urea (7), total nitrogen (8) and creatinine (9). Samples of finger capillary blood, obtained at the same times as the venous samples, were analyzed for glucose by the Somogyi-Nelson method (10).

In 18 instances the saline infusion was continued throughout the entire study, but at the onset of the first test period a single dose of glucagon[1] was administered intramuscularly or intravenously. In 12 of these 18 studies a highly purified, crystalline preparation of glucagon (0.5 or 1.0 mg.) was used; in 5, glucagon preparations of 50 per cent potency (compared to the crystalline preparation) in doses ranging from 1 to 7.5 mg.; and in 1, a heat-inactivated (100° C. for nine hours at pH 7) crystalline glucagon preparation.

In the remaining 7 subjects a constant infusion of 10 per cent dextrose in water was substituted for the isotonic saline during the test periods. The glucose infusion rate ranged from 255 to 477 mg. per minute in different subjects.

In 2 of the patients with gout, additional studies were made. The daily excretion of uric acid and creatinine in 12-hour collections of urine (7 A.M. to 7 P.M., and 7 P.M. to 7 A.M.) was followed before, during, and after several days of administration of glucagon in a dosage of 1 mg. of the crystalline preparation intramuscularly three times daily just before meals. These patients consumed a constant diet and received no other therapy throughout the studies.

RESULTS

Immediate effects of glucagon

Figure 1 illustrates the typical effects of a single injection of glucagon on the renal excretion of uric acid, total nitrogen and urea nitrogen, and on the renal clearance of creatinine in a normal subject. There was an immediate and sustained increase in the excretion of uric acid—maximum 117 per cent and mean 65 per cent greater than control values. Changes in total nitrogen and urea nitrogen paralleled each other and closely resembled changes in creatinine clearance, though slightly greater in magnitude.

Table 1 is a summary of data on the 14 normal subjects and 4 patients with gout who received a single dose of glucagon; 17 were given an active preparation, and 1 an inactive one. The data are expressed as the mean

[1] Generously supplied by Dr. W.R. Kirtley, Eli Lilly Co., Indianapolis.

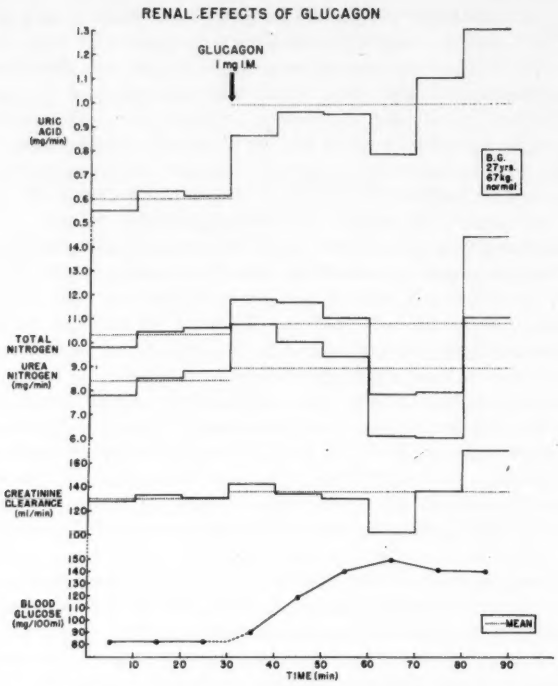

FIG. 1. Typical effects of a single injection of glucagon in a normal subject.

values for the 3 control periods and the mean values for the 6 periods of glucagon administration.

In 16 of the group receiving the active preparation, uric acid excretion increased significantly ($P < .001$) from 15 to 79 per cent (mean, 37 per cent). In the seventeenth subject (J.M.) there was a 20 per cent increase during the first two test periods, followed by a decrease below control levels in the remaining periods, with a mean net change of -7 per cent. In 14 of the patients the increase in uric acid excretion persisted throughout all test periods; in the remaining 3 the increase lasted for two to four periods only.

Total nitrogen and urea nitrogen excretions were measured in 5 of the normal subjects. Following administration of glucagon, changes in total nitrogen excretion varied from $+5$ to $+22$ per cent; the changes in urea nitrogen excretion (range, $+6$ to $+24$ per cent) closely paralleled those of total nitrogen. These increases were significant ($P = .01$).

The changes in endogenous creatinine clearance following administration of glucagon varied from -2 to $+16$ per cent (mean, $+5$) of control values. They showed a close relationship to the changes in total nitrogen

TABLE 1. RENAL AND PLASMA EFFECTS OF GLUCAGON

Subject, Age, Wt. & Diagnosis		Urine flow (ml./min.)	Creatinine clearance (ml./min.)	Urinary excretion (mg./min.)			Plasma level (mg./100 ml.)			
				Uric acid	Total N	Urea N	Uric acid	Total N	Urea N	Glucose*
M.H., 29,	Control	10.0	97	0.37	10.3	9.0	3.7	1077	14.9	79
60 Kg.,	Glucagon	12.3	113	0.65	12.7	11.1	3.6	1040	14.6	130
Normal	% change	+23	+16	+79	+22	+24	−3	−3	−2	+65
W.M., 45,	Control	8.2	97	0.57	7.5	6.8	6.8	1123	10.7	82
66 Kg.,	Glucagon	12.6	98	0.66	8.1	7.4	6.3	1089	10.7	125
Normal	% change	+53	+ 1	+17	+ 8	+10	−7	−3	0	+ 52
F.D., 54,	Control	4.7	78	0.27	10.0	8.6	6.0	1158	20.9	102
69 Kg.,	Glucagon	5.5	80	0.45	11.3	9.8	6.0	1107	20.6	157
Normal	% change	+16	+ 3	+67	+13	+13	0	−4	−1	+ 54
T.E., 27	Control	11.3	132	0.38	10.0	9.1	5.7	1066	10.7	75
82 Kg.,	Glucagon	11.7	136	0.52	11.6	10.4	5.7	1039	10.9	150
Normal	% change	+ 4	+ 3	+38	+16	+14	0	−3	+2	+100
B.G., 27,	Control	12.8	130	0.60	10.3	8.4	5.6	1138	11.7	81
67 Kg.,	Glucagon	8.1	136	0.99	10.8	8.9	5.3	1086	12.2	149
Normal	% change	−37	+ 4	+65	+ 5	+ 6	−5	−5	+4	+ 84
P.R., 35,	Control	11.0	124	0.66			4.9	1034	10.5	85
76 Kg.,	Glucagon	10.8	127	0.84			5.0	1023	10.4	129
Normal	% change	− 2	+ 2	+31			+2	−1	−1	+ 52
L.T., 30,	Control	12.5	131	0.64			5.6			86
79 Kg.,	Glucagon	10.8	138	0.86			5.3			151
Normal	% change	−14	+ 6	+35			−5			+ 76
C.C., 51,	Control	13.5	116	0.94			5.0			83
72 Kg.,	Glucagon	16.4	124	1.14			4.7			178
Normal	% change	+22	+ 8	+21			−6			+115
T.T., 33,	Control	11.0	116	0.41			5.2			83
78 Kg.,	Glucagon	11.0	118	0.47			5.1			158
Normal	% change	0.0	+ 1	+15			−2			+ 90
J.M., 46,	Control	14.5	125	0.67			4.2			
63 Kg.,	Glucagon	15.0	126	0.62			4.0			
Normal	% change	+ 4	+ 1	− 8			−5			
J.T., 26,	Control	14.2	96	0.44			4.7			
54 Kg.,	Glucagon	13.8	107	0.54			4.5			
Normal	% change	− 3	+11	+22			−4			
R.W., 22,	Control	15.3	97	0.53			5.5			
53 Kg.,	Glucagon	18.1	110	0.72			5.6			
Normal	% change	+18	+14	+37			+2			
T.O., 41,	Control	9.3	99	0.47			5.7			90
82 Kg.,	Glucagon	9.6	107	0.70			5.7			174
Normal	% change	+ 4	+ 8	+49			0			+ 93
H.C., 64,	Control	5.0	71	0.51			6.6			76
77 Kg.,	Glucagon	7.2	76	0.66			6.7			165
Gout	% change	+44	+ 7	+28			+2			+117
F.T., 48,	Control	7.2	130	0.52			5.6			88
63 Kg.,	Glucagon	8.8	128	0.68			5.6			155
Gout	% change	+22	− 2	+32			0			+ 76
C.S., 59,	Control	11.5	104	0.67			7.5			90
71 Kg.,	Glucagon	10.8	104	0.86			7.1			150
Gout	% change	− 6	0	+28			−5			+ 67
A.R., 60,	Control	5.7	65	0.33			12.0			76
88 Kg.,	Glucagon	4.9	69	0.41			12.1			143
Gout	% change	−14	+ 6	+22			+1			+ 88
W.M.,** 34,	Control	11.2	129	0.40			5.0			100
80 Kg.,	Glucagon	12.7	124	0.41			5.5			92
Normal	% change	+13	− 4	+ 3			+10			− 8

* In whole blood.

** Received inactivated glucagon.

TABLE 2. RENAL AND PLASMA EFFECTS OF GLUCOSE

Subject, Age. Wt. & diagnosis	Glucose infused i.v. (mg./min.)		Urine flow (ml./min.)	Creatinine clearance (ml./min.)	Urinary excretion (mg./min.)			Plasma level (mg./100 ml.)			
					Uric acid	Total N	Urea N	Uric acid	Total N	Urea N	Glucose*
J.H., 23, 76 Kg., Normal	334	Control	10.1	110	0.38	10.3	9.4	5.4	1054	14.9	90
		Glucose	8.9	103	0.36	9.6	8.5	5.5	1036	14.0	165
		% change	−12	− 7	− 4	−7	−9	+2	−2	−6.0	+83
W.C., 41, 78 Kg., Normal	326	Control	9.1	123	0.69	8.8	7.5	6.2	1174	11.3	88
		Glucose	8.4	121	0.68	8.6	7.5	6.2	1142	11.3	129
		% change	− 8	− 2	− 2	−3	0	0	−3	0	+47
S.L., 40, 65 Kg., Normal	329	Control	8.4	134	0.38	11.3	9.9	5.0	1120	12.7	74
		Glucose	8.2	130	0.37	11.6	10.0	5.0	1092	12.7	131
		% change	− 3	− 3	− 3	+3	+1	0	−3	0	+77
R.C., 48, 71 Kg., Normal	347	Control	11.4	100	0.30	9.0	8.2	4.0	1063	13.7	81
		Glucose	11.1	103	0.32	9.2	8.4	4.0	1051	13.7	126
		% change	− 3	+ 3	+ 8	+2	+3	0	−1	0	+56
F.F., 36, 92 Kg., Normal	413	Control	14.2	122	0.37			6.4			74
		Glucose	13.0	108	0.33			6.7			115
		% change	− 8	−11	−11			+5			+55
K.Q., 51, 78 Kg., Normal	477	Control	10.0	128	0.58			5.0			69
		Glucose	12.5	127	0.64			5.4			117
		% change	+24	− 1	+11			+8			+70
B.H., 34, 76 Kg., Normal	255	Control	11.0	86	0.49			6.5			99
		Glucose	13.8	86	0.49			6.6			132
		% change	+25	0	0			+2			+33

* In whole blood.

and urea nitrogen excretions but bore little relation to the changes in uric acid excretion. The increases in uric acid, total nitrogen and urea nitrogen were significantly ($P < .03$) greater than the increase in creatinine clearance.

Alterations in urine flow following administration of glucagon were not significant ($P = .13$) and bore no relationship to alterations in excretion of the nitrogenous substances. The changes in plasma levels of uric acid, total nitrogen and urea nitrogen following glucagon were small and variable. The maximum rise in the concentration of blood sugar following glucagon ranged from 52 to 117 per cent of control values.

The subject (W.M.) who received a heat-inactivated preparation of glucagon showed negligible changes in creatinine clearance, uric acid excretion and blood glucose concentration.

Neither the route of administration (intramuscular or intravenous) nor the dose (from 0.5 to 3.25 mg. or equivalent of the crystalline preparation) of glucagon appeared to have any influence on the direction or magnitude of the changes observed.

Immediate effects of glucose

Table 2 contains a summary of data on 7 normal subjects who served as a control group for the glucagon studies. They received a constant intravenous infusion of 10 per cent glucose in water following 3 control periods, and their blood sugar patterns were similar to those of the subjects who received glucagon.

The excretions of uric acid, total nitrogen and urea nitrogen were virtually unchanged during administration of glucose. The changes in endogenous creatinine clearance varied from −11 to +3 per cent of control values, and in urine flow from −12 to +25 per cent of control values. The changes in plasma concentrations of uric acid, total nitrogen and urea were minor and inconstant. The maximum rise in blood glucose concentration varied from 33 to 83 per cent of control values.

Effects of prolonged administration of glucagon

Figure 2 shows the effect of glucagon, administered three times daily, on the 12-hour excretion of uric acid and creatinine in one of the patients with gout. Glucagon had no significant effect on the day or night excretion of uric acid or creatinine. Similar results were obtained in another patient with gout.

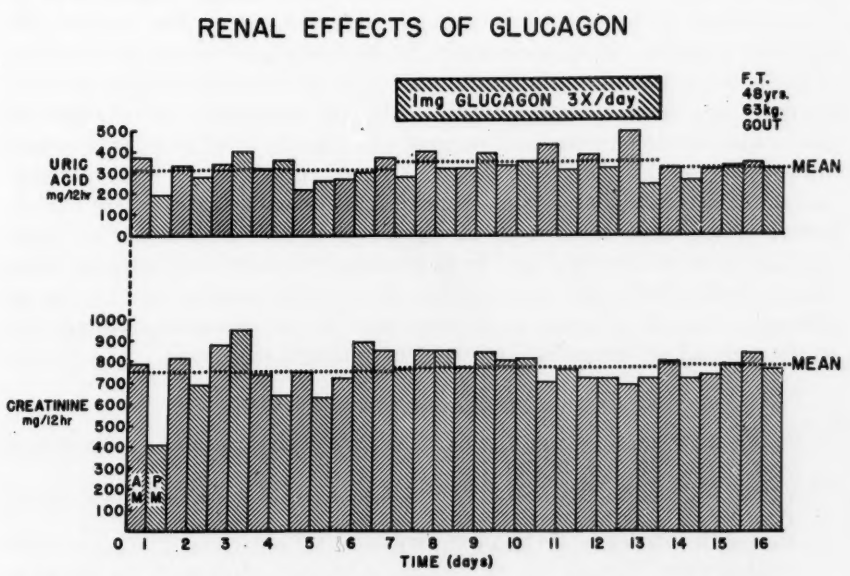

FIG. 2. Effect of glucagon administered three times daily in a patient with gout.

DISCUSSION

The present studies show that glucagon causes an immediate and significant increase in the renal excretion of uric acid in normal subjects and in patients with gout. The duration of this effect is at least one hour in most cases. However, no significant change in the 12-hour urinary excretion of uric acid was detected when glucagon was administered to 2 patients with gout who had previously shown a significant, immediate uricosuric response to a single injection of glucagon. The immediate uricosuric effect is not due to the hyperglycemic action of glucagon, since glucose-induced hyperglycemia of similar magnitude did not increase uric acid excretion. Since the uricosuric effect was observed with highly purified crystalline glucagon and not with a biologically inactivated preparation, it is probable that the effect was due to the glucagon molecule itself.

The changes in total nitrogen and urea nitrogen following administration of glucagon, though significant, were small, and paralleled the variations in creatinine clearance. For this reason they were probably due only to changes in the glomerular filtration rate. Glucagon caused a small but consistent increase in glomerular filtration rate, and this supports previous observations (4, 5).

The mechanism of the immediate uricosuric action of glucagon is unknown. It is probably due to a renal tubular effect, though a glomerular or hemodynamic action cannot be completely ruled out on the basis of the available evidence. It appears not to be part of a general catabolic action of glucagon, as postulated by Salter *et al.* (11), because the changes in total nitrogen and urea excretion were small and paralleled the changes in glomerular filtration rate, and because the plasma level of total nitrogen always decreased. Salter *et al.* observed a marked increase in total nitrogen and urea excretion (11) as well as an elevated basal metabolic rate (12) in rats receiving large doses of glucagon by injection. Helmer *et al.* (13) mention increases in uric acid and total nitrogen excretion in 3 patients with rheumatoid arthritis given a ten-hour intravenous infusion of 12.5 mg. of glucagon. This discrepancy in findings may be due to species difference or to the marked difference in the dosage of glucagon employed.

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ACTIVITY OF IODOTYROSINE DESHALOGENASE IN "NORMAL" AND DISEASED HUMAN THYROIDS*

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ABSTRACT

Iodotyrosine deshalogenase assays were performed on 40 surgical specimens from 39 human thyroid glands. Histologically normal thyroid tissue from 8 patients with thyroid cancer served as "normal" controls. The iodotyrosine deshalogenase activity of thyroid tissue from 10 patients with colloid adenomatous goiter and 5 patients with Hashimoto's struma was within the range of activity found in "normal" thyroid tissue. Iodotyrosine deshalogenase activity was significantly lower ($P < 0.02$) than "normal" in the thyroid glands of 2 patients with nonspecific thyroiditis. A clearcut difference from "normal" activity was not found in the thyroid glands of 8 thyrotoxic patients rendered euthyroid with propylthiouracil and Lugol's solution. The Michaelis-Menten constant for iodotyrosine deshalogenase in the thyroids of these thyrotoxic patients did not differ significantly from "normal" (2.3×10^{-5} *M* and 1.8×10^{-5} *M*, respectively). The mean iodotyrosine deshalogenase activity in carcinomatous tissue from the thyroids of 8 patients was less than "normal" and tended to be lower in undifferentiated thyroid carcinoma than in the well differentiated form. The normal iodotyrosine deshalogenase activity found in Hashimoto's struma, despite numerical reduction in follicles, is consistent with the hypothesis of an increased effect of TSH in this disorder. Because of preoperative preparation of the thyrotoxic patients with iodine, failure to find a clearcut increase above normal deshalogenase activity in the thyroids of this group cannot be considered evidence against the hypothesis of an increased action of TSH in thyrotoxicosis. The findings in thyroid carcinoma are consistent with a correlation between loss of differentiation and loss of function.

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IODOTYROSINE deshalogenase catalyzes the removal of iodine and bromine from tyrosine but not from thyronine (1). Appreciable amounts have been found in some but not in all human (2) and animal (2, 3) organs, the thyroid being especially rich in this enzyme. Congenital absence of the enzyme can result in goitrous cretinism (4). In animals pretreated with thyrotropic hormone (TSH), the activity of thyroidal iodotyrosine deshalogenase is increased (1). To investigate further the importance of this enzyme in human thyroid function, we determined deshalogenase activity in 40 samples of thyroid tissue from 39 patients with various thyroid diseases.

MATERIAL AND METHODS

Histologic and clinical material

Forty surgical specimens of thyroid glands from 39 patients were analyzed for iodotyrosine deshalogenase activity. Sections of all thyroid glands were examined grossly and microscopically by a pathologist. An additional histologic control was obtained in all but 3 patients by microscopic examination of a slice of tissue taken directly from the precise area which was being assayed simultaneously for enzyme. Tissues classified as "normal" were grossly and histologically normal areas of thyroid gland from patients in whom the primary diagnosis was carcinoma of the thyroid elsewhere in the gland. The histologic classification of the thyroid tissues studied included 7 "normal," 10 colloid adenomatous goiter, 8 treated exophthalmic goiter, 5 Hashimoto's struma, 2 chronic nonspecific thyroiditis, and 8 carcinoma. In a few instances, human lymph node tissue from a subject without thyroid disease, and serum from patients with colloid adenomatous goiter or metastatic thyroid cancer were similarly analyzed.

The patients listed as thyrotoxic in Figures 1 and 2 and Table 1 were so classified on the basis of clinical and laboratory findings present before thioamide drugs and stable iodine were given in preparation for surgery. At the time of operation, all were clinically euthyroid but the results of thyroid function tests suggested that 6 were euthyroid and 2 were still mildly hyperthyroid.

All other patients were reviewed carefully for evidence of hypo- or hyperthyroidism. Three patients who did not undergo any thyroid function tests were euthyroid clinically. In 23 clinically euthyroid patients, the diagnosis was confirmed by at least one thyroid function test. Among the remaining 5 clinically euthyroid patients, results of thyroid function tests indicated mildly increased function in 4 and decreased thyroid function in 1, at the time of operation.

In all patients, within approximately five minutes (usually two minutes) after surgical removal, the thyroid tissue was frozen in a vial immersed in a flask containing a mixture of dry ice and alcohol and stored at $-40°$ to $-60°$ C.

For analysis, the tissues, while still frozen or just beginning to thaw, were cut freehand with a scalpel into thin slices. Enough slices to weigh 150 mg. (± 10 per cent) were placed in each of several (preferably 5) incubation flasks. As the enzyme is somewhat unstable, slicing was expedited by using a constant amount of tissue in all incubation flasks, but the amount of substrate was varied. Monoiodotyrosine, both labeled with I^{131} (0.5 μc. per flask) and unlabeled, was used as substrate. Each flask contained Krebs-Ringer phosphate buffer (pH 7.1 to 7.2) plus the sucrose-electrolyte solution described by Stanbury (5), approximately 10^{-3} *M* thiouracil, 5×10^{-2} *M* nicotinamide and 0.6 mg. of triphosphopyridine nucleotide (TPNH). In each flask the volume of sucrose-electrolyte

solution was 0.6 ml.; the combined volume of aqueous solutions used to introduce the co-factors and thiouracil was 0.45 ml., and the final volume after addition of Krebs-Ringer phosphate was 4.0 ml. The slices were brought to 37° C. and incubated, with shaking, in a Dubnoff metabolic incubator for twenty minutes. When sufficient tissue was available, 5 flasks containing the following amounts of substrate were incubated: 20 (±4), 35 (±6), 51 (±6), 101 (±10) and 200 (±10) millimicromoles per flask. Although in several instances the complete five-point analysis could not be run because of shortage of tissue, we were able to run a four-point analysis per tissue on the average in the overall study.

A flask containing boiled slices from the same tissue was run under identical conditions as a control in each experiment. The reaction was stopped at the end of incubation by adding an excess of non-labeled monoiodotyrosine and iodide to all flasks and boiling the mixture for forty-five seconds. Aliquots of the incubates were transferred to small tubes and applied quantitatively to No. 3 Whatman filter paper strips for descending chromatography in a butanol:acetic-acid:water system. The counts per minute applied to the paper were determined by counting the radioactivity in the aliquot before application to the paper and the residual activity in the micropipettes at the end of application. After about ten hours in this chromatography system, the strips were removed, dried at room temperature, and cut into 1-cm. sections. Each section was counted in a well-type scintillation counter with the aid of a pulse-height selector. The areas corresponding to free and tyrosine-bound iodine were regularly and easily recognizable as peaks, with the appropriate Rf and staining reactions. The deshalogenase activity was calculated according to the following formula:

$$A = 3\,S\left[\frac{\left(\frac{TU}{TC}\right)(MC) - MU}{\left(\frac{TU}{TC}\right)(MC)}\right]$$

where

A = deshalogenase activity (millimicromoles of substrate deiodinated per hour). The amount of deiodination obtained in a 20-minute incubation period is converted to the amount of deiodination per hour by introducing the factor of 3 in the foregoing formula.

S = amount to substrate (millimicromoles).

TU = total counts per minute in the entire chromatography strip for the unknown material.

TC = total counts per minute in the entire chromatography strip for the control boiled material.

MC = counts per minute in the monoiodotyrosine area of the control strip.

MU = counts per minute in the monoiodotyrosine area of the unknown strip.

As a check on the efficiency of the chromatography, the effective recovery on each strip was calculated by adding the counts per minute in the liberated iodine and monoiodotyrosine areas and dividing this sum by the total counts per minute applied to the paper. The mean recovery was $\geq$80 per cent in 35 of the tissues and 70–80 per cent in the remaining 5. A few tissues yielding a mean recovery of less than 70 per cent were discarded from this series and were *not* included in the foregoing 40.

Comparisons between activity in slices and activity in homogenates of the same gland were carried out on tissue from a few benign and a few malignant thyroids. No definite difference in enzyme values between homogenate method and slice method was observed.

RESULTS

Figure 1 shows the results in benign thyroid tissue. The activity in colloid adenomatous goiter and in Hashimoto's struma is probably in the "normal" range. The results in thyrotoxicosis and chronic nonspecific thyroiditis as compared with "normal" tissue are shown in more detail in Table 1 and Figure 2. Comparison of the curves for chronic nonspecific thyroiditis and "normal" tissue by application of the "*t*" test at each substrate concentration shows significantly lower activity for the former ($P < .01$ for each of the lowest 3 substrate concentrations; $.02 > P > .01$ for each of the remaining concentrations). Similar comparison of the curves for thyrotoxic and "normal" tissue fails to establish significant differences, except at the 51-millimicromole substrate level ($P = .05$). In the absence of any real difference between the two types of tissue at any of the 5 sub-

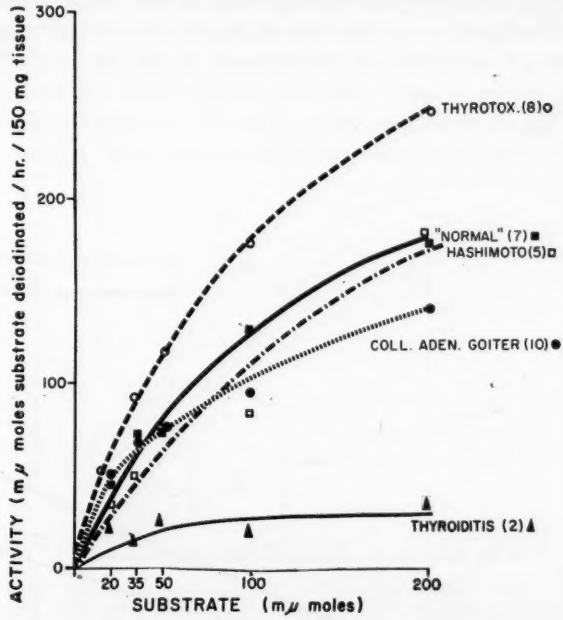

FIG. 1. Iodotyrosine deshalogenase activity of benign human thyroids. Each curve was obtained from the mean values of iodotyrosine deshalogenase activity, with increasing amounts of substrate, for one diagnostic category; the experimentally determined data points from which the curves were obtained are also given. The diagnostic categories are listed at the right end of each curve. Each diagnosis is followed by a figure in parentheses, giving the number of such tissues studied to derive these data. The symbol to the right of this number designates which symbol has been used for the data points experimentally determined from thyroid tissue in that diagnostic category.

TABLE 1. IODOTYROSINE DESHALOGENASE ACTIVITY IN "NORMAL" AND DISEASED THYROID GLAND TISSUE

Type of thyroid tissue	No. of patients	Enzyme activity (millimicromoles deiodinated/hr.) at substrate concentrations (millimicromoles/4 ml.) of:				
		20	35	50	101	200
"Normal"	5	42 (±6)*	70 (±13)	74 (±16)	129 (±31)	176 (±41)
Thyrotoxicosis	8	51 (±3)	92 (± 5)	115 (±10)	175 (±40)	247 (±56)
Chronic nonspecific thyroiditis	2	23	17 (±12)	24	20 (±20)	35 (± 7)

* Mean and Standard Error of the Mean.

strate concentrations, the probability that a difference of at least this magnitude might occur by chance alone at some one of the 5 concentrations is, $1-(0.95)^2$ or slightly greater than 1 chance in 5. Combination of the "t" values for all substrate concentrations, as described by Herdan (6), yields results which suggest that the whole curve for thyrotoxicosis is significantly higher than the whole curve for "normal" tissue ($P < .05$ by this test). However, when the data for thyrotoxic and "normal" glands are

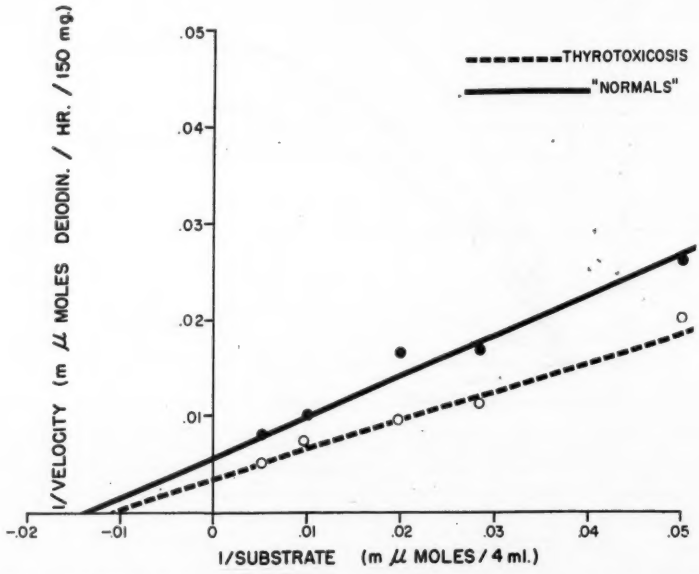

FIG. 2. Iodotyrosine deshalogenase activity of "normal" and thyrotoxic human thyroids. The data are plotted according to the method of Lineweaver and Burk.

plotted according to the method of Lineweaver and Burk (7) (Fig. 2), comparison of the slope by the "t" ("F" test) (8) fails to reveal a significant difference ($0.25 > P > 0.1$), and comparison of ordinate intercepts by the same test also fails to reveal a significant difference ($0.5 > P > 0.25$). The equation for the regression line for "normal" tissue is $1/V = 0.423(1/s) + 0.00576$ and for thyrotoxicosis, $1/V = 0.307(1/s) + 0.00342$. The Michaelis-Menten constants are 1.8×10^{-5} M and 2.3×10^{-5} M for "normal" and thyrotoxic tissues, respectively. As this constant is determined by the slope and ordinate intercept in the Lineweaver and Burk plot, the difference in Michaelis-Menten constants is also not significant.

The results in the 8 cancers studied are compared with the mean "normal" values in Figure 3. A positive correlation between the degree of differentiation and the deshalogenase activity was suggested by our results. For example, the tumor showing the highest activity (Patient B.S.) was a well differentiated follicular adenocarcinoma, whereas the tumor showing complete absence of activity (Patient A.M.) was the least differentiated thyroid carcinoma studied. Nevertheless, the tendency of thyroid cancer to

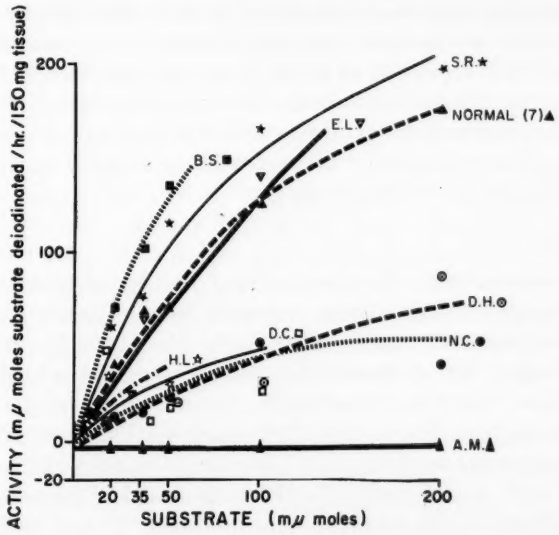

FIG. 3. Iodotyrosine deshalogenase activity of "normal" and malignant human thyroids. Each curve was obtained from the iodotyrosine deshalogenase activity values for a single patient, except the curve presenting the mean values in 7 "normal" thyroid tissues. The experimentally determined data points from which the curves were obtained are also given. The symbol to the right of the patient's initials designates which symbol has been used for the data points experimentally determined from that patient's thyroid tissue.

surround and isolate nests of apparently normal follicles and also to be associated with a lymphocytic reaction made it difficult to obtain "pure" cancer for analysis.

In the sample of human lymph nodes from a subject without thyroid disease, deshalogenase activity was very low—less than 10 millimicromoles per hour even at the highest substrate concentration. The samples of serum from a patient with metastatic carcinoma of the thyroid (E.L., whose thyroidal activity is shown in Figure 3) and from a patient with colloid adenomatous goiter (also tested at two, four and a half, and twenty-four hours after administration of 10 units of thyrotropin (Armour) intramuscularly) exhibited no significant deshalogenase activity.

DISCUSSION

These studies allow certain quantitative comparisons between enzyme activity in "normal" and in diseased human thyroid tissue. When feasible, this approach may be more direct than the study of disorders artificially created in laboratory animals.

It is recognized that actual incubation for one hour would not be expected to yield precisely the same results as those reported by our present method of calculation, in which the results of a twenty-minute incubation are multiplied by 3. However, as all our data were obtained in twenty-minute incubation periods and all were multiplied by the same factor, comparison within our data remains strictly valid. Furthermore, the expression of our results in terms of activity per hour should simplify comparison with other data already in the literature.

Thyrotoxicosis

Querido and associates (2) reported that the deshalogenase activity in thyrotoxicosis (determined in only 1 gland), though slightly higher than in nodular goiter, was not significantly so. At that time the technique involved incubation for six hours. Co-factors were not added. Following Stanbury's more recent modification (5), we shortened the incubation time and added co-factors. Even with these modifications, we did not find a clearcut difference between "normal" and thyrotoxic thyroid tissue. Evaluation by the well established "t^2" ("F") test failed to reveal a significant difference. Results of the less elaborate modified "t" test as described by Herdan (6) suggested a difference, but this test is less reliable in an experimental design in which each thyroid gland contributes slices for analysis at several enzyme concentrations, than in a design in which each gland is used for only one substrate concentration. The "t^2" ("F") test suffers much less from this disadvantage and is therefore more useful here.

As TSH has been shown to increase iodotyrosine deshalogenase activity in animals, the apparent lack of difference in the activity of this enzyme

between thyrotoxic and "normal" thyroid tissue may have important implications. We were unable to obtain exophthalmic goiter tissue for analysis from a patient who had received no preoperative preparation, but the possible influence of preoperative preparation must be considered. The preoperative administration of iodine had resulted in a marked decrease in the follicular cell mass of the thyroid in the patients with exophthalmic goiter. In most instances only an occasional epithethial spur and some increased lymphocytic infiltration were present as evidence of previous thyrotoxicosis. Even though TSH has been shown to increase iodotyrosine deshalogenase activity (per gram of wet tissue) in the thyroid gland of animals, this effect may merely depend upon the increased cellular mass of a gland that has been rendered hyperplastic. Thus, even though the effect of TSH is usually increased in untreated thyrotoxicosis, iodotyrosine deshalogenase activity is not necessarily increased if the hyperplasia of the toxic gland has been reversed by iodine. For this reason, our findings need not be interpreted as refuting the hypothesis of an increased effect of TSH in untreated human thyrotoxicosis. Propylthiouracil is not likely to decrease iodotyrosine deshalogenase activity; Roche found that the thyroids of rats pretreated with propylthiouracil showed, if anything, slightly higher iodotyrosine deshalogenase activity than normal.

If clinical hypothyroidism *per se* decreases the activity of this enzyme (9, 10), the question arises as to whether or not deviation from the euthyroid state could have affected the results in our patients. Six of our 8 thyrotoxic patients, however, had been brought to a euthyroid state at the time of operation. None was hypothyroid at the time of operation and 2 were mildly hyperthyroid, their preoperative basal metabolic rates (BMR) being +14 and +19 per cent. The mean deshalogenase activities of thyroid tissue in the latter 2 patients, at the 5 substrate levels, were 45, 89, 119, 128 and 230. Thus, if these 2 cases were omitted from the group, the mean results in thyrotoxic patients would be higher rather than lower. It seems unlikely, therefore, that deviations from the euthyroid state affected our values.

The Michaelis-Menten constant for the iodotyrosine deshalogenase content of "normal" human thyroid is approximately twenty times greater than that found by Stanbury (5) for sheep thyroid.

Hashimoto's struma and chronic nonspecific thyroiditis

The normal values for deshalogenase activity in Hashimoto's struma are somewhat surprising. In these patients, as in the patients with chronic nonspecific thyroiditis, the number of follicles per unit volume of tissue is considerably reduced, as shown by histologic sections. Nonfollicular tissue represents a large portion of each specimen. Yet, in contrast to the decrease in activity seen in chronic nonspecific thyroiditis, the iodotyrosine

deshalogenase activity in Hashimoto's struma is within the normal range. The low deshalogenase activity that we have found in human lymph nodes and that Roche *et al.* (1) have found in dog's spleen suggests that lymphatic tissue, which is the principal nonthyroid tissue in Hashimoto's struma, contributes very little to deshalogenase activity. The results indicate that the activity per thyroid follicle is increased in Hashimoto's struma. This is consistent with the hypothesis that TSH activity is increased in Hashimoto's struma (11).

The finding of normal values for iodotyrosine deshalogenase activity in Hashimoto's struma and subnormal values in chronic nonspecific thyroiditis does not necessarily imply that these disease states have different causes. Hashimoto's struma may conceivably represent an early stage, and chronic nonspecific thyroiditis a later stage, of the same disease.

Nontoxic colloid adenomatous goiter

Since in 3 of the patients with colloid adenomatous goiter there was evidence suggesting that minimal hyperthyroidism was present at the time of thyroidectomy, the question arises as to whether or not iodotyrosine deshalogenase activity in truly euthyroid colloid adenomatous goiters may actually be subnormal. In 1 of these 3 clinically euthyroid patients the serum protein-bound iodine level was 9 μg. per 100 ml., and the BMR was +3 per cent. The second patient had a 24-hour thyroidal I^{131} uptake of 43 per cent, and a BMR of +11 per cent. The third patient had borderline clinical evidence of hyperthyroidism. If the values for iodotyrosine deshalogenase activity in these patients are subtracted from the values for all the patients with colloid adenomatous goiter, the curve of the mean remaining values would be slightly higher than before. It seems unlikely, therefore, that undiagnosed mild hyperthyroidism had falsely elevated the values for iodotyrosine deshalogenase activity in this type of nontoxic goiter.

Carcinoma of the thyroid

It is unlikely that clinical hypothyroidism in association with thyroid carcinoma accounted for the subnormal iodotyrosine deshalogenase activity observed in the carcinomatous tissues, since all but 1 of the patients with thyroid carcinoma were euthyroid at the time of thyroidectomy. It was not unexpected to find that iodotyrosine deshalogenase activity was subnormal in thyroid carcinoma, and that the more undifferentiated carcinomas showed the least activity. The relatively small amount of I^{131} taken up by thyroid carcinoma constitutes additional evidence that in the development of the carcinoma, loss of differentiation is accompanied by loss of function.

The rough *inverse* correlation between the activity of iodotyrosine des-

halogenase and the ratio of cell mass to colloid mass in cancers cannot be taken as good evidence that colloid is the principal source of this enzyme. Loss of colloid and loss of enzyme activity may both simply be parallel manifestations of the degree to which the cancer has degenerated from normalcy. Moreover, of all diseased tissues, cancers may well be the least representative of normal enzyme physiology. The increased iodotyrosine deshalogenase activity reported in animals given TSH suggests, on the other hand, a *direct* correlation between the activity of iodotyrosine deshalogenase and the ratio of follicular cell mass to colloid mass. However, even this evidence, which seems more pertinent than the opposing evidence from cancer, is not conclusive. The parallel occurrence of two effects after administration of TSH does not prove that one of the effects caused the other.

Acknowledgments

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EXPERIENCE WITH THE *IN VITRO* ERYTHROCYTE UPTAKE OF I^{131}-LABELED *l*-TRIIODOTHYRONINE IN A ROUTINE CLINICAL LABORATORY*

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ABSTRACT

The *in vitro* erythrocyte uptake of I^{131}-labeled *l*-triiodothyronine was determined reproducibly in a routine clinical laboratory 424 times in 348 subjects. The erythrocyte uptake was significantly lower in euthyroid females than in euthyroid males. Hyperthyroidism caused a significant elevation, whereas hypothyroidism, menstruation, pregnancy and liver disease caused a significant depression of the uptake. The administration of iodine-containing compounds was associated with an increase in the erythrocyte uptake, but not sufficient to interfere with the usefulness of the test. This test is an inexpensive simple and rapid method of estimating thyroid function; the administration of radioisotope is eliminated. The results compare favorably in accuracy with those of other currently used thyroid function tests, and may be more useful for purposes of following the effects of therapy in thyroid disease, and in the management and diagnosis of normal and disordered pregnancy.

IN 1957, Hamolsky, Stein and Freedberg (1) described the *in vitro* erythrocyte uptake of I^{131}-labeled *l*-triiodothyronine (T_3) as an outgrowth of their studies of the plasma protein-hormone complex in thyroid physiology (2, 3). Crispell *et al.* also reported their results with the procedure (4, 5). More recently, Hamolsky and his associates reviewed the data of 2900 determinations carried out in their laboratory (6). The present study was undertaken to assess the applicability of this procedure to conditions in a routine clinical laboratory and to the management of clinical problems.

MATERIALS AND METHODS

The test was originally performed (1) by adding the labeled hormone in duplicate to 3-ml. samples of blood in Erlenmeyer flasks, which were then rotated for two hours in

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a water bath at 37° C. From each flask, 1.0 ml. was taken in duplicate and the radioactivity measured before and after washing the erythrocytes in saline. For use in a routine laboratory, the procedure has been modified as follows:

1) A 5.0-ml. sample of blood is drawn (fasting not necessary) into mixed oxalate.[1] The test is performed within forty-eight hours to avoid significant lysis of the erythrocytes. Duplicate 1.0-ml. aliquots of whole blood are pipetted into Pyrex tubes (15×100 mm.).
2) A 0.05-ml. portion of a stock solution of chromatographically pure I^{131}-labeled *l*-triiodothyronine dissolved in propylene glycol[2] is withdrawn with a tuberculin syringe and diluted with sufficient physiologic sodium chloride to yield 3×10^4 c.p.m. per 0.1 ml., by the following formula:

   $\mu c./ml.$ of stock solution $\times 0.133 \times$ decay factor = ml. of sodium chloride solution.
3) To each tube is added 0.1 ml. of the diluted I^{131}-labeled T_3.
4) The tubes are then shaken in a water bath at 37° C. for two hours in an apparatus consisting of an Eberback variable rotator to which a metal bar is attached, suspending a rubber rack in the water bath.
5) The total radioactivity in each tube is measured in a well-type scintillation counter.[3]
6) After centrifugation, the supernatant plasma is removed, and the erythrocytes washed five times with tenfold volumes of isotonic sodium chloride solution.
7) The radioactivity remaining in the erythrocyte fraction is then similarly counted.
8) The "erythrocyte uptake" is calculated as follows:

$$\frac{\text{Net counts in washed erythrocytes} \times 100 \text{ hematocrit}}{\text{Net counts in whole blood} \times \text{patient's hematocrit}} \times 100 = \%.$$

   The uptake is corrected to a hematocrit of 100 for purposes of comparison. The results of duplicate determinations are averaged; if the difference between duplicates is greater than 3 per cent uptake, the results are not reported.

Tests were performed on personnel, patients who were scheduled for thyroid studies (thyroidal I^{131} uptake, serum protein-bound iodine level, basal metabolism), and other unselected patients having a variety of medical diseases. Blood samples were obtained from the patients of a local obstetrician (cases of doubtful pregnancy, threatened and suspected abortion, and menstrual disorders). Thyroid status was determined independently of the results of *in vitro* studies.

RESULTS

This study was conducted over a seventeen-month period in a clinic run by 4 internists and provided with a well-equipped, small, routine clinical laboratory; 424 determinations were performed on 348 adults (32 subjects whose clinical status could not be determined were not included). The relationship between erythrocyte uptake of I^{131}-labeled T_3 and the functional status of the patient is shown in Table 1.

[1] Ammonium oxalate (2.4 Gm.) and potassium oxalate (6.0 Gm.) in 200 ml. of water.

[2] Obtained from Abbott Laboratories, Oak Ridge, Tennessee. Specific activity: 20–50 millicuries per milligram of *l*-triiodothyronine.

[3] The samples are counted for a sufficient period of time to obtain a counting error of less than 1%, with correction for background radiation.

TABLE 1. EFFECT OF THYROID STATUS ON THE *in vitro* ERYTHROCYTE UPTAKE OF I^{131}-LABELED *l*-TRIIODOTHYRONINE

Thyroid status	No. of patients	No. of tests	Range (%)	Mean ± S.D. (%)
Euthyroid (all patients)	272	300	10.1–23.0	15.4±2.24
Male	120	133	11.9–23.1	16.3±2.4
Female	152	167	10.1–19.1	14.6±1.93
Thyrotoxic	12	15	17.1–26.4	21.3*
Hypothyroid (all patients)	53	57	6.4–16.2	12.1±1.5
Male	13	14	9.8–16.2	12.6*
Female	40	43	6.4–15.6	11.9±1.3
Euthyroid in association with recent administration of iodine	11	20	13.5–22.0	16.2±2.10

* The small series did not justify statistical analysis of variance.

Euthyroidism. In euthyroid men, the erythrocyte uptake was significantly higher than in euthyroid women. Therefore, the mean for each sex and the overall means for the 300 observations in the euthyroid group, were used in further calculations.

Hyperthyroidism. The total of 15 observations was small, but the average value was significantly different from that in the euthyroid group. With improvement in the thyrotoxic state, one patient's uptake decreased from 25.0 per cent to 18.7 per cent, and another's from 19.6 per cent to 14.9 per cent. Although only 4 determinations were made on thyrotoxic women, the values (mean, 18.9 per cent) were lower than the 11 values (mean, 22.2 per cent) obtained for thyrotoxic men. The highest value (26.4 per cent) was in the most severely toxic patient, a woman. The level of erythrocyte uptake seemed to parallel the degree of toxicity.

Hypothyroidism. The average uptake was significantly lower than in euthyroid subjects. Again, the mean value for males was higher than that for females. One woman was frequently tested; it is of interest that clinical hypothyroidism developed while she was under observation and there was a good response to thyroid medication. Her erythrocyte uptakes, shown in Table 2, also demonstrate the effect of menstruation. Another patient who was examined before induction of myxedema by radioiodine for treatment of angina pectoris, was followed during the development of hypothyroidism and the response to thyroid therapy (Table 3).

Previous iodine therapy. The mean erythrocyte uptake following admin-

TABLE 2. *In vitro* UPTAKE OF I^{131}-*l*-TRIIODOTHYRONINE BY THE ERYTHROCYTES OF A PATIENT IN WHOM HYPOTHYROIDISM DEVELOPED WHILE UNDER OBSERVATION

The effects of hypothyroidism, thyroid therapy, and menstruation are demonstrated.

Date	Erythrocyte uptake (%)	Clinical status
1957		
Oct. 21*	12.6	Euthyroid
Nov. 1	15.3	Euthyroid
6	15.1	Euthyroid
18*	12.1	Euthyroid
Dec. 16*	12.0	Euthyroid
1958		
Jan. 10	13.1	?
Apr. 29*	10.2	?
July 16	12.5	Hypothyroid
Oct. 8	10.8	Hypothyroid (6-hr. thyroidal uptake of I^{131}, 4.4%; 24-hr., 10.9%)
1959		
Jan. 7	15.4	Euthyroid (taking thyroid, 2 grains daily)
June 3	14.7	Euthyroid (taking thyroid, 2 grains daily)

* Menstruation.

istration of iodine was significantly higher than in the euthyroid group, but not enough higher to prevent the test from being useful, especially since the other thyroid tests (serum protein-bound iodine level and thyroidal I^{131} uptake) are so markedly affected. In 3 of the 11 subjects, thyroidal uptake of I^{131} was determined simultaneously and demonstrated the expected depression.

TABLE 3. *In vitro* UPTAKE OF I^{131}-*l*-TRIIODOTHYRONINE BY THE ERYTHROCYTES OF A PATIENT IN WHOM MYXEDEMA DEVELOPED WHILE UNDER OBSERVATION

The effects of hypothyroidism and thyroid therapy are demonstrated.

Date	Erythrocyte uptake (%)	Clinical status	Daily dose of thyroid (grains)
1957			
Nov. 20	15.1	Euthyroid	0
1959			
Jan. 12	10.6	Myxedema	0
Jan. 20	12.0	Hypothyroid	¼
Feb. 2	9.8	Hypothyroid	¼
Feb. 11	14.0	Mildly hypothyroid	½
June 3	15.8	Euthyroid	1½

Hashimoto's struma. Two patients, one euthyroid and one hypothyroid by other tests, were examined. In each the erythrocyte uptake was 11.4 per cent.

Nontoxic nodular goiter. Six patients (3 males and 3 females) were studied. The erythrocyte uptakes ranged from 13.8 to 16.8 per cent (average, 15.6 per cent). These patients were euthyroid when tested by other procedures, and the findings did not differ significantly from those for the euthyroid group.

Acromegaly. One man, euthyroid on the basis of other observations, had an erythrocyte uptake of 17.4 per cent.

Hypopituitarism (chromophobe adenoma). One man, hypothyroid on the basis of all other observations, had an erythrocyte uptake of 10.4 per cent.

Estrogen therapy. Estrogen was given to 3 women before the erythrocyte uptake was determined. The uptakes were 13.2, 14.4 and 9.1 per cent.

Menstruation. It was observed, early in the study, that the erythrocyte uptake was low during menstruation. Data on 5 patients are listed in Table 4. Eighteen determinations were made on 13 women during menstruation. The uptakes ranged from 8.5 to 15.0 per cent (average, 11.9 per cent).

Pregnancy. During pregnancy there was a pronounced decrease in the erythrocyte uptake. Six pregnant women were studied; the data on 3 of these are listed in Table 4. Patient B was seen before and during pregnancy; Patients F and G were followed during and after pregnancy. The 12 uptake values during pregnancy ranged from 6.8 to 12 per cent (average, 9.3 per cent). One patient aborted the day following study; her uptake was 9.6 per cent. There was a strong suspicion that the abortion was induced. One patient, known to be an habitual aborter, had an erythrocyte uptake of 13.0 per cent; she was delivered after only six months of gestation; the baby died. The time at which the erythrocyte uptake becomes depressed during pregnancy is not known. Two subjects were studied in the fourth week of pregnancy and the erythrocyte uptakes were 11.9 and 13.8 per cent. Thus it seems that by the second month of pregnancy the uptake is consistently depressed.

Liver disease. Two patients were studied during attacks of hepatitis; the man had uptakes of 13.7 and 16.0 per cent, and the woman had an uptake of 11.4 per cent. In a woman dying of hepatic failure associated with metastatic breast cancer, the uptake was 10.4 per cent. Another woman with chronic hepatitis had an uptake of 9.5 per cent. The average value of the 5 determinations in the presence of liver disease was 12.2 per cent.

Hematocrit. In confirmation of the findings of Hamolsky *et al.* (6), there was a reduction in erythrocyte uptake with decreasing hematocrit readings. It is suggested, therefore, that before a determination of uptake is

TABLE 4. THE EFFECT OF MENSTRUATION AND PREGNANCY ON THE ERYTHROCYTE UPTAKE OF I^{131}-*l*-TRIIODOTHYRONINE

(Successive determinations on each patient are given in descending order.)

Patient A	Patient B	Patient C	Patient D	Patient E	Patient F	Patient G
15.5	13.1*	14.3	15.3	13.0†	8.0 (4 mo. preg.)	12.0 (8 mo. preg.)
14.8	15.4	14.2	12.6*	8.7*	7.9 (5 mo. preg.)	9.3 (9 mo. preg.)
						Delivered at term, 2 wks. later
13.3*	11.2*	11.9*	15.1		6.8 (6 mo. preg.)	
	10.0 (2 mo. preg.)		12.1*		9.4 (8 mo. preg.)	17.5
					Delivered at term	
	7.8 (5 mo. preg.)		12.0*			
	Delivered at term				13.2	
					13.8	

* Menstruation.

† Menstruation began 12 hours later.

made on blood with an hematocrit of 35 per cent or less the balance be corrected by withdrawal of plasma, since the uptake drops below 80 per cent of the original value after this point.

Statistical evaluation: Tests of significance were carried out by calculating "*t*," utilizing the formula for independent means. These calculations were made for the first 383 determinations. The results are given in Table 5.

DISCUSSION

With the recognition of the importance of thyroid dysfunction of mild degree, and the increasing trend toward routine examinations in industry and the population at large, laboratory procedures have taken on more im-

TABLE 5. LEVELS OF SIGNIFICANCE (t) BETWEEN MEANS OF VARIOUS GROUPS

Groups	Euthyroid			Hypothyroid	
	female	male	total	female	total
Euthyroid					
female		16.2***			
male	16.2***				
Thyrotoxic					
male		8.1***	17.5***		
total			14.8***		
Hypothyroid					
female	13.0***		11.3***		
male		6.5***	9.4***		
total			18.2***		
Iodine therapy					
female	3.0*		1.2		
male		0.97	3.8**		
total			10.0***		
Thyroid therapy					
female	7.0***		6.6***	1.7	
total			7.3***		1.7
Liver disease			7.1***		0.12
Nontoxic nodular goiter			1.2		3.5*
Menses	9.5***		14.4***	0.66	
Pregnancy	14.6***		19.2***	5.6***	

* Significant at 1% level.
** Significant at 0.1% level.
*** Significant beyond the 0.1% level.

portance and screening procedures have been given wider application. Some cases are diagnostic problems; indeed, all available tests may not be sufficient to make a diagnosis. There are many parameters of thyroid function, and measuring only one of them provides a distinctly incomplete picture, just as a single liver function test or an electrocardiogram would provide an incomplete picture of liver or cardiac function, respectively (7). Moreover, all of the present procedures have features which limit their use-

fulness. Therefore, any test which measures an additional parameter of thyroid function is welcome, and deserves applications to clinical problems.

Thyroid metabolism, in particular the protein-binding of thyroid hormones, has been discussed in a number of recent papers (8–15). In this test, it would seem that the plasma content of thyroxine-binding globulin and its affinity for the added labeled triiodothyronine determines the amount of the hormone remaining to be "taken up" by the erythrocytes. The criss-cross experiments reported by Hamolsky *et al.* (1) demonstrated that erythrocyte uptake is determined by the thyroidal state of the patient from whom the plasma is obtained. Results of studies with saline suspensions of erythrocytes, to which plasma was added, support this theory (4, 5). Moreover, studies of the thyroxine-binding capacity of the plasma indicate an increased binding in patients with myxedema, correlated with a decreased erythrocyte uptake. Likewise, a decreased binding capacity is found in patients with hyperthyroidism, correlated with an elevated erythrocyte uptake (16). The decreased erythrocyte uptake during pregnancy is associated with an increase in specific binding globulins of plasma (17–20). An excellent discussion of the physiology involved and the theoretical difficulties with the too-ready acceptance of this attractive theory is presented by Hamolsky *et al.* (6).

The literature includes many observations on the relationship of thyroid function to menstruation, pregnancy, and female sex hormones. On the whole, the results have been variable and difficult to correlate (21–24). The erythrocyte uptake of I^{131}-T_3 is decreased during pregnancy. Our results also indicate a decrease during menstruation. This may prove to be a fertile area for further study in order to clarify some of the seemingly contradictory evidence. The suggestion of Hamolsky and associates (6) that miscarriages may be forecast by the erythrocyte uptake procedure is provocative. Our results on this point are limited, but thus far support their view.

It is possible, by means of the *in vitro* erythrocyte uptake of I^{131}-T_3 to assess simply and rapidly the thyroid function of most patients, in a routine clinical laboratory. The test is, therefore, useful as a rapid screening method. The accuracy compares favorably with that of other currently used methods (7, 25). It avoids the administration of isotope to the patient (an advantage during pregnancy, for example) and is little influenced by administration of iodine, in contrast to the determination of the protein-bound iodine level and the thyroidal uptake of I^{131}. The procedure is useful in following the response to therapy in hyperthyroidism and hypothyroidism, and in gauging its adequacy. In some instances, the results obtained with this test correlate better with the thyroid status of the patient than do results obtained with other methods. Thus, it appears that the *in*

vitro erythrocyte uptake of I^{131}-T_3 may be of diagnostic value in cases in which certain factors complicate the interpretation of other thyroid diagnostic procedures.

Problems exist, though, in the use of this method. Erythrocytes are fragile, and the test must be carried out relatively soon after the blood is withdrawn if lysis is to be prevented. Moreover, a more definite understanding of the meaning of the test is necessary in order to be certain that it truly measures thyroid function.

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CHEMICAL DETERMINATION OF ALDOSTERONE IN URINE

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ABSTRACT

A method for the determination of aldosterone in urine is presented, which includes the following steps: acid hydrolysis and extraction, column chromatography on silica gel, 2 paper chromatographic systems, acetylation, a third paper chromatographic system, and fluorometry directly on the paper using alkaline fluorescence. Data are presented on precision, recovery, duration of acid hydrolysis, acid versus enzymatic hydrolysis, stability of urine samples and normal values.

THE importance of aldosterone in the regulation of water and salt metabolism has made highly desirable the availability of a specific chemical method of reasonable accuracy for its determination. Of the published physico-chemical procedures, at least 2 appear to have the necessary specificity and accuracy, namely, the method of Ayres, Garrod, Simpson and Tait (1) and that of Nowaczynski, Koiw and Genest (2). The method of Nowaczynski and coworkers was set up in our laboratories for routine analyses. One urine specimen was encountered, however, in which substitution of acetylation and the Bush B3 system for the Bush B5 system employed as the third paper in Nowaczynski's method yielded considerably lower results. This led to a study of the method, and the introduction of some modifications of the technic of Nowaczynski which are believed to increase the yield and simplify the quantitation in the final step. Essentially, these modifications consist of (a) an increase in the time of hydrolysis, (b) acetylation and substitution of the Bush B3 system for the Bush B5 system used by Nowaczynski for the final separation of aldosterone on paper, and (c) fluorometric estimation by carrying out the Bush alkaline fluorescence reaction directly on paper, a procedure used by Ayres, Simpson and Tait (3). A detailed description is given of the technics used, with data on reproducibility, effect of varying the time of hydrolysis, recovery of known standards, normal values, and stability of urine samples.

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METHOD

Reagents

1. Silica gel, 60–200 mesh, preactivated. Davison Chemical Co., Baltimore, Md.

2. Whatman No. 2 filter paper, 46×57 cm. sheets. Each sheet is cut into 3 equal parts, yielding strips of about 15×57 cm. The strips are rolled up, placed in a 1500-ml. capacity Soxhlet extractor (J-1565(G), Scientific Glass Apparatus Co., Inc., Bloomfield, N. J.) and extracted for seventy-two hours with methanol, A. R. grade. The strips are air dried and serrated at one end by pinking shears.

3. Purified methanol. Methanol is purified as follows: To a liter of absolute methanol add 0.5 Gm. of 2,4-dinitrophenylhydrazine hydrochloride and 0.5 ml. of concentrated hydrochloric acid. Let stand in the dark at room temperature overnight. Distill through a Vigreaux column, discarding the first 75–100 ml. Collect the portion boiling at 65° C. Discontinue the distillation when a yellowish condensate is observed on the thermometer bulb. Repeat the distillation.

4. Acetone-chloroform, 1+99, both A. R. grade.

5. Acetone-chloroform, 1+1, both A. R. grade.

6. Ethylene glycol-methanol, a 50 per cent (v/v) solution of ethylene glycol (EK No. 133) in absolute methanol, A. R. grade.

7. Solvent for the first paper system—ethylene glycol-toluene. Approximately 50 ml. of ethylene glycol (EK No. 133) are added to 2 liters of toluene (A. C. S. grade) and the mixture shaken for about four minutes. Only the upper toluene phase is used, so the mixture can be stored in a large bottle and, as the toluene is used up, more toluene can be added.

8. Solvents for the second paper system—Eberlein-Bongiovanni (E_2B). Let the following mixture stand for one hour at 37° C.: 167 ml. of 2,2,4-trimethylpentane (EK P2396), 150 ml. of distilled water and 83 ml. of tert-butanol (EK 820). Separate the 2 phases.

9. Solvents for the third paper systems—Bush B3. Shake the following mixture for about four minutes: 45 ml. of distilled water, 180 ml. of purified methanol, 150 ml. of heptane (EK 2215) and 75 ml. of benzene (A. C. S. grade, redistilled). After shaking, keep at room temperature for several hours and then separate the 2 phases.

10. Blue tetrazolium (BT) reagent, 0.1 per cent aqueous solution. This reagent is stable for one week in the refrigerator. Blue tetrazolium obtained from General Biochemicals, Inc., Chagrin Falls, Ohio, proved to be very satisfactory.

11. Methanolic-KOH solution. KOH, 14 per cent (w/v) in 60 per cent (v/v) purified methanol.

12. Alkaline BT reagent. Mix 1 volume of reagent #10 with 9 volumes of reagent #11 immediately before use.

13. Reference standards. (a) Cortisol and cortisone. A solution is made up in purified methanol so that a 20-μl. portion contains approximately 5 μg. of each steroid. Concentration need be only approximate, since these steroids are used only as qualitative reference guides. (b) Aldosterone. When first setting up the method it is necessary to have a pure sample of aldosterone for standardization. Solutions in purified methanol are employed. Either the free form or *d,l*-aldosterone-21-monoacetate can be used, since standardization is begun with the acetylation step before the third paper system. The authors are grateful to Dr. J. M. Jailer, Presbyterian Hospital, Columbia University, New York City, and to Ciba Pharmaceutical Products, Summit, N. J., for an initial supply of aldosterone for standardization. If only a limited supply of pure reference

aldosterone is available, the steroid may be isolated from third-trimester pregnancy urine and standardized against the pure reference material. The acetylated aldosterone can be recovered from the third paper system and can be further purified by rerunning on the third paper system. If free aldosterone is desired, the third paper system employed in the method presented here (Bush B3) should be replaced by the Bush B5 system (2) and the acetylation step omitted.

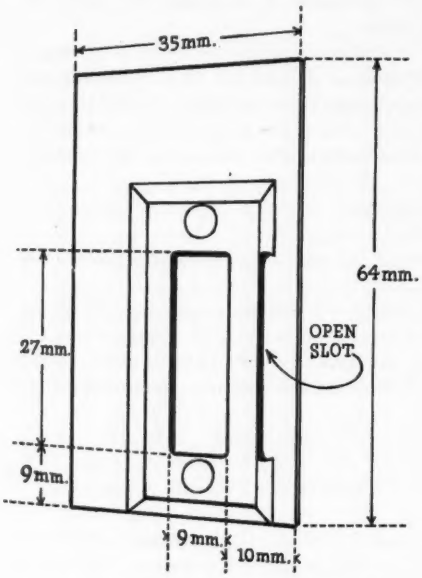

FIG. 1. Apparatus for holding filter paper, when reading fluorescence.

Equipment for fluorometry

The fluorometer used was a Photovolt, Model 540, with a photomultiplier (Photovolt Corp., N. Y. 16). The primary filter employed was a Photovolt B-HG-1 with a peak transmittance at 365 mμ and the secondary filter was a Klett 56 with a peak transmittance at 560 mμ.

Spot fluorescence was read directly on the filter paper while holding the paper in a special holder inserted in the fluorometer cuvet compartment at a 45° angle. The design of the holder is shown in Figure 1. It can be made of heavy black or blackened cardboard or of metal with a dull black finish. Black paints which fluoresce must not be used.

Procedure

1. *Hydrolysis and extraction.* Half of a 24-hour urine collection is adjusted with concentrated hydrochloric acid to pH 1, using pH paper, and extracted continuously with chloroform equal in volume to half the urine sample. A convenient apparatus for this procedure consists of a 2-liter or 4-liter separatory funnel with a mechanical stirrer holding a double-flanged glass rod to agitate both layers gently. Four changes of solvent are made during the 48-hour extraction period, the last being a hand shakeout. A Cohen-type extractor (4, 5) can be used, as previously suggested (1, 2), but it is not very practical. This apparatus cannot be left unattended overnight because of chloroform evaporation.

2. *Alkali wash and evaporation.* The pooled chloroform extracts are washed twice with one-twentieth volumes of 0.1 *N* NaOH. The alkali layer is back-extracted with an equal volume of chloroform. The combined chloroform extracts are then washed twice with one-twentieth volumes of distilled water and the washings similarly back-extracted with chloroform. The combined chloroform extracts are dried with anhydrous sodium sulfate. The solvent is distilled off under vacuum (water pump) using a nitrogen leak, at 40–45°C. Use of a rotary evaporator (2) was found to be unsatisfactory because of contamination from the rubber and grease used in the apparatus.

3. *Chromatography on silica gel.* A silica-gel column is prepared by making a slurry composed of 3 Gm. of silica gel in 5 ml. of chloroform and pouring it into the column

(1×18 cm.). (If a large residue is obtained from step 2, it is advisable to use 2 or 3 silica-gel columns in order not to overload these micro-columns; one column should be used for no more than about 15 mg. of residue.) The silica-gel column is covered with a layer of glass wool. The dried sample is transferred quantitatively to the column in several 2-ml. portions of chloroform. A 50-ml. portion of acetone-chloroform (1+99) is passed through the column and discarded. A 100-ml. volume of acetone-chloroform (1+1) is used for elution. The eluate is evaporated under nitrogen in a water bath at 45°C.

4. *First paper system.* Descending chromatography is used throughout. Two or 3 sheets (15×57 cm.) of Whatman No. 2 paper are used, the number being dependent upon the amount of residue from the previous step; the decision is easily made with experience. Usually 2 papers will suffice, but putting too much on one paper results in overloading and poor separation. A horizontal line is drawn 12 cm. below the non-serrated edge of each paper to serve as the starting line. The papers are dipped into a 50 per cent solution of ethylene-glycol in methanol, then drained and blotted between 2 sheets of clean filter paper to remove excess stationary phase. The paper is then dried in air for about fifteen minutes. The dried sample residue is dissolved in 100 μl. of purified methanol and, by means of a 25-μl. micropipet, is streaked along the starting line, beginning 3.5 cm. inward from one long side and extending for 8 cm. The sample tube is rinsed with 100 μl. of solvent, which is also applied to the sample streak. Reference spots containing approximately 5 μg. each of cortisone and cortisol in purified methanol are applied 2 cm. inward from each edge. (A useful device to help in applying solutions to paper is a sample applicator such as Model S 1250 of Research Equipment Corp., Oakland, California). The paper is then mounted in the chromatographic tank (a Chromatocab Model A 300, Research Equipment Corp., was used) and equilibrated for four hours over toluene. The paper is developed with toluene saturated with glycol, for forty-eight hours at room temperature. At the conclusion of the run, the paper is dried either in a forced-draft oven or in a fume hood for one hour at room temperature. The paper is then photographed and processed as follows: Pin it down over photographic paper (R-3 Ansco) and expose to a short-wave ultraviolet lamp (1 second with Mineralight lamp, Model NH, Ultra Violet Products, San Gabriel, California, held 40 cm. above the paper). After development of the photograph, which is used as a guide, cut out that section of the chromatographic paper which extends from just below (below with reference to the descending pattern) the slower-moving cortisol band to the upper part of the faster-moving cortisone band. Cut the section up into pieces measuring about 1 sq. cm., transfer to a 100-ml. Erlenmeyer flask and cover with 10 ml. of purified methanol. Place in a water bath at 45°C. for ten minutes, with occasional swirling. Decant liquid through a funnel (containing a glasswool plug) into a 35-ml. glass-stoppered centrifuge tube. Repeat extraction of the paper with three more 10-ml. portions of methanol. Evaporate off the solvent under nitrogen in a water bath at 45°C. Dissolve residue in 1 ml. of methanol, add 4 ml. of water and extract three times with 3-ml. portions of chloroform (A. R. grade). This step serves to separate the steroid from the ethylene glycol which is present in the paper extract. Transfer the chloroform extracts to a 12-ml. or 15-ml. conical centrifuge tube and evaporate to dryness under nitrogen in a water bath at 45°C. The residue is transferred to the second paper as described subsequently.

5. *Second paper system.* Whatman No. 2 paper is cut to dimensions and ruled as just described. The sample is applied along a 6-cm. section of the starting line. Only one paper is used unless very prominent bands appear in the aldosterone region of the first papers. A reference spot of cortisone and cortisol is also applied. The paper is equilibrated in a tank, containing both separated phases of the E_2B System, for six hours at room

temperature. The chromatogram is developed, using the upper solvent phase, for twelve hours. The paper is then dried at room temperature in a fume hood or forced-draft oven. The paper is photographed under ultraviolet light as described previously, and that section of the chromatographic paper extending from the lower edge of the cortisol band to a line 6 cm. above the upper edge of this band is cut out. This section is extracted with methanol as previously described, and the methanol is evaporated off. The residue is quantitatively transferred with purified methanol to a 12-ml. or 15-ml. conical centrifuge tube and the methanol evaporated off.

6. *Acetylation.* The residue from step 5 is acetylated as follows: To the residue add 0.2 ml. of pyridine (A. R. grade) and 0.1 ml. of acetic anhydride (A. R. grade), stopper tightly with a cork stopper, and let stand overnight at room temperature. Evaporate to dryness in a water bath at 45°C. under nitrogen. Evaporate repeatedly with 0.2-ml. quantities of purified methanol until no odor of pyridine remains. The residue is now ready for the third paper. An aldosterone standard (5 μg., calculated as free aldosterone, is a convenient amount) is also dried and acetylated at this time.

7. *Third paper system.* Whatman No. 2 paper is cut and ruled as described. If the photographic copy of the second paper shows no band or only a faint band in the aldosterone position, then the residue of acetylated steroid is dissolved in 25 μl. of purified methanol and applied as a spot to the starting line. Spots should be round, and about 5 mm. in diameter. The residue is washed out again with an additional 25 μl. of solvent, which is applied to the same spot. The acetylated standard is dissolved in 25 μl. of solvent and applied in a similar manner. If the photograph reveals a prominent band in the aldosterone position, then the residue of acetylated steroid is dissolved in 100 μl. of solvent, and a 25-μl. portion (equivalent to a quarter of the sample) is applied as a spot to the starting line, followed by an additional 25 μl. of solvent. The remaining solution of the residue is evaporated to dryness, the residue dissolved in 25 μl. of solvent, and the entire amount (equivalent to three quarters of the sample) applied as an additional spot on the same paper in the manner described previously. Since the solvent front of the Bush B3 system moves fairly rapidly, the rate of flow is slowed down by fixing a second glass anti-siphon rod about 1 inch horizontally from the usual rod resting in the cradle and passing the paper over both rods. The paper is then equilibrated in a tank containing both phases of the Bush B3 system for four hours at room temperature and developed with the upper solvent phase for six hours at room temperature. The paper is dried at room temperature in a fume hood or a forced-draft oven. It may be photographed (UV) for a permanent record.

8. *Development of fluorescence.* Sufficient alkaline BT reagent is poured into a glass tray (8×10 inches) to form a shallow layer. A glass rod is attached to each end of the paper by clips. By grasping these rods, the paper is held taut and lowered over the tray until the bottom side of the paper is just wetted by the solution. The paper is removed from contact with the liquid (the starting end of the paper being slightly elevated so that excess fluid is drained off) and it is held in a horizontal position over a warm-air blower until dry. The paper is then heated in a draftless oven for twenty minutes at 60–65°C. The paper is examined under the ultraviolet lamp and the fluorescent spots due to aldosterone outlined with pencil. If a fluorescent spot is deformed, this is evidence of incomplete separation, and the third paper should be rerun. Once it is decided to proceed with quantitation, the center of the fluorescent spot is marked with a pin hole and the spot excised so that the paper fits the special holder and so that the pin hole coincides with the center of the aperture of the holder. A fluorescence reading is made on each side, the mean value being used for calculation. The "blank fluorescence" of the paper is determined similarly on a piece cut from the area preceding the starting line.

Calculation:

$$\frac{\text{sample fluorescence} - \text{blank fluorescence}}{\text{standard fluorescence} - \text{blank fluorescence}} \times \mu\text{g. of aldosterone in standard} \times 2 = \mu\text{g. of aldosterone per 24-hour output.}$$

EXPERIMENTAL

Relation between amount of aldosterone and fluorescence reading

Ayres, Simpson and Tait (3) reported that the intensity of fluorescence was proportional to the amount of aldosterone present if the spot contained less than 4 μg. and was less than 2 cm. in diameter.

Several experiments were run with varying amounts of acetylated aldosterone on the third paper system. All final spots were approximately 1.5 cm. in diameter. Deviation from linearity between fluorescence readings and amounts of aldosterone began to appear at about 7.5 μg. of aldosterone. The data indicated, however, that no great error would be incurred if linearity is assumed up to 10 μg.

Precision

Fourteen analyses were performed on 6 urine samples, the aldosterone content of which ranged from 5 to 100 μg. per twenty-four hours. The 95 per cent confidence limits (8 D.F. $\pm 2.31s$) were ± 22.5 per cent.

Recovery of aldosterone added to urine

When free aldosterone in amounts varying from 7 to 11 μg. was added to urine before the hydrolysis step, the recoveries in 6 different runs ranged from 69 to 81 per cent, with a mean of 74 per cent. When aldosterone was added to the chloroform extract after hydrolysis, the recoveries in 4 different experiments ranged from 77 to 84 per cent, with a mean of 80 per cent.

Recovery of aldosterone as a function of the period of hydrolysis

Tait (6) reported the data of one experiment using a 48-hour period of acid hydrolysis and indicated that only 70 per cent of the total aldosterone obtained was split off during the first twenty-four hours, the remainder being liberated by the second 24-hour period of hydrolysis.

Four different urine samples were analyzed; 3 were late pregnancy urines, and the fourth a urine from a case of nonpregnancy hyperaldosteronism. Two of the specimens were subjected to hydrolysis for seventy-two hours, and 2 for forty-eight hours. At the end of each 24-hour period the chloroform extracts were removed and processed, the residual urine being further subjected to hydrolysis for additional 24-hour periods. Each 24-

hour extract was carried through the entire procedure and the aldosterone was measured quantitatively. The results are shown in Table 1.

The finding of a very significant percentage of the total recoverable aldosterone in each extract from the second 24-hour hydrolysis is evidence that a 48-hour period of hydrolysis is required for satisfactory recovery. The negligible amounts of the steroid found in the third 24-hour extract of samples A and B suggest that a 72-hour period is not required.

Acid versus glucuronidase hydrolysis

Previous reports (7, 8) have indicated that acid hydrolysis at pH 1 is more efficient than hydrolysis by glucuronidase. In the present study, the 2

TABLE 1. EFFECT OF DURATION OF HYDROLYSIS ON RECOVERY OF ALDOSTERONE FROM URINE

Period of hydrolysis	Aldosterone in urine (μg./24 hrs.)			
	Urine sample A	Urine sample B	Urine sample C	Urine sample D
First 24 hours	60	61	45	17
Second 24 hours	44	34	15	14
Third 24 hours	0	1.6	—	—
Total	104	96.6	60	31

methods of hydrolysis were compared, using 1 normal and 2 pregnancy urines. Acid hydrolysis yielded 24-hour aldosterone excretion values of 4.0, 64 and 77 μg., and enzymatic hydrolysis with Ketodase (Warner-Chilcott) yielded values of 2.2, 1 and 3.3 μg. respectively.

Stability of aldosterone in urine

Male and female urines samples were collected and pooled according to sex. One portion of each was frozen immediately after collection and stored for ten days in the frozen state. A second portion of each was held at room temperature without any preservative for five days. To a third portion of each, chloroform was added (10 ml. per liter), and the urine was then held at room temperature for seven days. An additional urine sample from a woman in the late stage of pregnancy was analyzed immediately after collection and also after standing at room temperature, with 10 ml. chloroform per liter, for a period of four days. To the samples held for determination of stability was added a mixed culture of bacteria from an alkaline urine specimen. Samples preserved with chloroform remained acid. The un-

preserved samples became alkaline. Results are shown in Table 2. It is presumed that to preserve urine with chloroform, one should be certain that the urine is acid to begin with.

Normal values

Determinations were performed on 5 adult males and 5 adult females. There was no statistically significant difference between sexes, so the results were pooled. The range of aldosterone excretion was 2 to 15 μg. per twenty-four hours. The data were normally distributed after log transformation and the 95 per cent limits obtained by the "normal equivalent deviate" method (9) were 2 to 23 μg. per twenty-four hours.

TABLE 2. STABILITY OF ALDOSTERONE IN URINE

Urine sample	Sex of patient	Preservative	Temp. of storage	Days of storage	Aldosterone (μg. per liter)
A	M	None	−20°C.	10	12
		None	Room temp.	5	3.4
		$CHCl_3$	Room temp.	7	10
B	F	None	−20°C.	10	10
		None	Room temp.	5	7.4
		$CHCl_3$	Room temp.	7	12
C	F	None	—	0	104
		$CHCl_3$	Room temp.	4	97

DISCUSSION

The question of specificity of paper chromatographic methods, such as the one described here, has been discussed by Nowaczynski, Koiw and Genest (2). Several comments concerning the specificity and accuracy of the method presented seem to be pertinent: (*a*) Quantitation by alkaline fluorescence was chosen because it has been established that it possesses greater specificity than quantitation by ultraviolet spectrophotometry or by photometry with blue tetrazolium reagent (10). Dinitrophenylhydrazine reacts with ketones, saturated as well as unsaturated, in the α,β position. Although increased specificity has been sought by use of correction formulas (11) when employing spectrophotometric methods, it seems preferable to employ a reaction which appears to be fundamentally more specific and does not require such corrections. (*b*) The specificity, for some urines at least, can be increased by being alert for certain warning signs such as incomplete separation of bands in the first 2 paper systems and deformed spots on the third paper system. Either one of these signs is

a good indication that a re-run on the particular paper system involved is advisable. This occurs only infrequently and the extra chromatographic step involves, in our experience, an additional loss of approximately 3 per cent. (*c*) In several hundred urines run thus far by the method presented, no evidence of blue fluorescence signifying the presence of compound III (12, 13) has ever been observed on the third paper system. (*d*) The time of hydrolysis has been extended to forty-eight hours in order to increase the yield from conjugates. Recoveries of free aldosterone added before hydrolysis were somewhat less than when added after hydrolysis, indicating some loss during the hydrolysis step. This is in agreement with the findings of Neher and Wettstein (14), who reported that free aldosterone is not stable for periods longer than twenty-four hours at pH 1. Considerably more is gained than is lost, however, by increasing the period of hydrolysis. (*e*) The work of Albeaux-Fernet and Romani (15) indicates that a significant fraction of conjugated aldosterone may not be split by acid hydrolysis.

The normal values obtained in this study are somewhat higher than those reported by other workers who employed methods yielding similar recoveries (1, 2, 14). This can be explained at least in part by the greater yield obtained by the longer period of hydrolysis employed in the method presented. The normal range obtained is in close agreement with that obtained by Ayres and coworkers (1). This most likely is coincidental, since they used hydrolysis for only twenty-four hours, but "partially corrected" for overall losses in the method.

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ENDOCRINE SIGNIFICANCE OF SHORT METACARPALS

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ABSTRACT

Observations on 2594 outpatients indicate that shortness of the fourth metacarpals relative to the third and especially relative to the fifth (metacarpal sign) is of frequent occurrence in patients with gonadal dysgenesis having either female or male sex chromatin. Moreover, in males (Klinefelter's syndrome excluded), this metacarpal sign occurs much more frequently in the presence of some gonadal anomaly than when the gonads are normal. Among 105 males exhibiting the metacarpal sign and 136 others exhibiting only the borderline condition, all of those from whom buccal smears were taken were found to have male sex chromatin. In all 5 males with female sex chromatin, there was no metacarpal sign. On the basis of limited data, it appears that the metacarpal sign, when found in more than one generation of the same family, is without significance as far as gonadal development in any generation is concerned. However, when it is found in only one generation, it is frequently associated with gonadal aberration. The converse also is true. Patients having the metacarpal sign usually have delayed skeletal development. Though an occasional one is taller than average, the mean height is appreciably less than the average expected height based on the skeletal age. The deviations from average in the relative lengths of metacarpals which result in the metacarpal sign, occur both more frequently and to a greater extent in the left hand than in the right, but do not appear to be the result of hand dominance.

ON A roentgenogram of the human hand, when a line is drawn tangentially to the circumference of the heads (distal ends) of the fourth and fifth metacarpals, the extension of this line usually passes distal to the head of the third metacarpal, as indicated in Figure 1 (negative metacarpal findings). In some hands, such a test line is tangential also to the circumference of the head of the third metacarpal as in Figure 2 (borderline metacarpal findings). In others, the line runs through the distal end of the third metacarpal as in Figure 3, indicating a specific relationship (metacarpal sign). In this study, the 2594 patients who attended the outpatient department of the child growth-study and endocrine clinics of the Rockefeller Hospital during the past ten years, have been classified according to whether or not they exhibited the metacarpal sign in either or both hands.

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FIG. 1

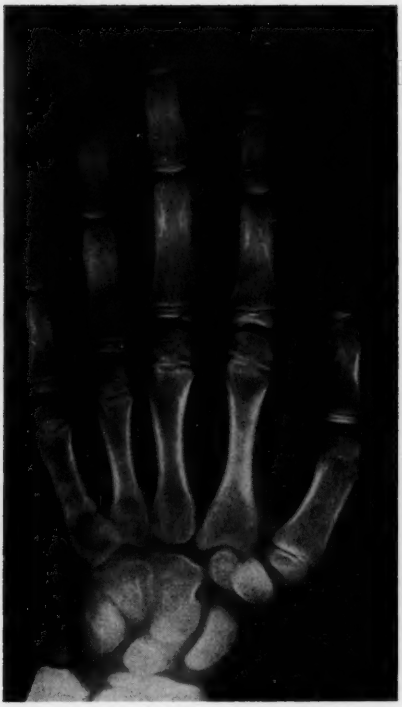

FIG. 2

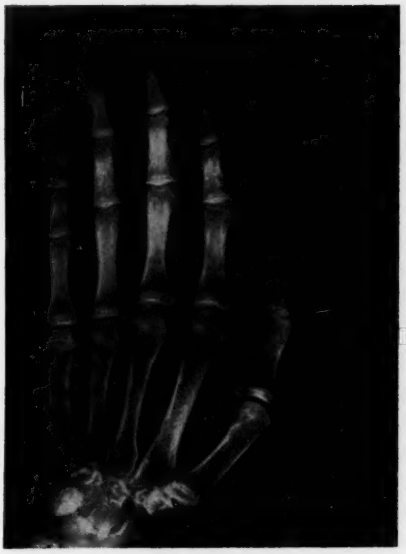

FIG. 3

FIG. 1. Roentgenogram of left hand and wrist of a 13.3-year-old boy with Klinefelter's syndrome. This illustrates the usual relationship of the metacarpals, regardless of whether or not Klinefelter's syndrome is present. The pattern was similar in the right hand.

FIG. 2. Roentgenogram of left hand and wrist of an 11.8-year-old boy whose testes had descended spontaneously at the age of 12.5 years. This illustrates a borderline metacarpal relationship, which was similar in the right hand.

FIG. 3. Roentgenogram of left hand and wrist of a 16.9-year-old girl with gonadal dysgenesis (Case #1, Table 3). This illustrates the metacarpal sign, which was present also in the right hand.

METHODS

The presence of a positive or a borderline finding was originally detected in the clinic by examination of the clenched fists. A pencil placed on the skin over the ends of the fourth and fifth metacarpals of a hand with fully flexed fingers, missed the third metacarpal in most subjects but touched the skin over the head of the third metacarpal in most of those in whom the sign was definitely present. X-ray examination is not necessary for clinical detection of this sign in some patients, but it was used in the present study, partly to observe the effect of aging on development of the phenomenon, and partly to permit inclusion in the study of patients who had been relocated or for other reasons could not return to the clinic.

With the patient seated, the hands were radiographed together. The hands were placed flat on a cassette resting on a table; the central ray was equidistant between the two hands and at the level of the metacarpal heads.

TABLE 1. DISTRIBUTION OF POSITIVE, BORDERLINE AND NEGATIVE METACARPAL FINDINGS (RIGHT AND LEFT HANDS) BY SEX

Sex	Metacarpal findings																			
	[1] R+, L+*		[2] R+, LB		[3] R+, L−†		[4] L+, RB		[5] L+, R−		[6] RB, LB		[7] RB, L−		[8] LB, R−		[9] R−, L−		[10] Totals	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Male	47	2.4	7	.35	13	0.7	11	0.6	27	1.4	44	2.2	49	2.5	43	2.2	1754	87.9	1995	76.9
Female	21	3.5	1	.17	8	1.3	7	1.2	6	1.0	9	1.5	7	1.2	21	3.5	519	86.6	599	23.1
Total	68	2.6	8	.31	21	0.8	18	0.7	33	1.3	53	2.0	56	2.2	64	2.5	2273	87.6	2594	100.

* R+, L+ means metacarpal findings positive in both right and left hands.
† RB, L− means metacarpal findings borderline in right hand and negative in left hand.

RESULTS

A summary of results is presented in Table 1. From these data it can be calculated that a positive or borderline finding was present in 12 per cent of the males and 13 per cent of the females in one or both hands, *i.e.*, the difference in sex incidence was insignificant. The positive metacarpal relationship was found in only 5.7 per cent of the patients and in only 4.1 per cent of the hands (for in only 2.6 per cent of the subjects was it found bilaterally). The condition was borderline in 7.7 per cent of the patients and in 4.8 per cent of the hands. The borderline condition was found with approximately equal frequency in the left and right hands (columns 2 and 8 as compared with columns 4 and 7). When the metacarpal sign was present, it was observed more frequently in the left hand than in the right (column 2 *vs.* 4, and column 3 *vs.* 5) When the finding was positive in one hand only, it was found 51 times on the left (columns 4 and 5) as compared with 29 times on the right (columns 2 and 3). Even when the findings were negative in both hands, the distance from the tangential line to the edge of the third metacarpal head was usually (though not invari-

ably) less in the left hand than in the right. Furthermore, when the metacarpal sign was present in both hands the test line usually cut deeper into the head of the third metacarpal in the left hand than in the right.

It has been shown that the position of the hand relative to the x-ray equipment and film is not the factor which determines which hand demonstrates the more distinct sign.

Assuming that the relative position of the proximal ends of the metacarpals is constant, the metacarpal sign can arise from shortness of the fourth metacarpal relative to either the third or to the fifth metacarpal or to both the third and the fifth. Although the definition of positive and negative metacarpal findings makes no mention of the position of the proximal ends of these metacarpals, the metacarpal sign is usually associated with shortness of the fourth metacarpal relative to the third and the fifth.

TABLE 2. RATIO OF LENGTH OF METACARPALS #3, 4 AND 5 IN PERSONS WITH POSITIVE AND NEGATIVE METACARPAL FINDINGS

Metacarpals findings	Ratio of lengths of metacarpals		
	#3 re #4	#3 re #5	#4 re #5
Positive	1.16	1.18	1.02
Negative	1.13	1.21	1.07

In the positive cases, metacarpal #4 was shorter relative to #3 than in the negative cases; #5 was longer relative to #3 than in the negative cases; #4 was much shorter relative to #5 than in the negative cases.

This was determined by measurement of the length of the x-ray images of the third, fourth and fifth metacarpals of the left hands of: a) 10 randomly selected female patients with bilateral positive findings and b) 10 randomly selected female patients with bilateral negative findings. The data, summarized in Table 2, show that in hands exhibiting the metacarpal sign, the fourth metacarpal is shorter relative to the third than in hands without the sign, and the shortness is even more marked relative to the fifth metacarpal. Indeed, in hands exhibiting the metacarpal sign the fifth metacarpal is larger relative to the length of the third than in hands without the sign. Examination of the original data shows that development of the metacarpal sign is due to 2 factors which usually operate simultaneously, *viz*, a more rapid growth of the third metacarpal and especially of the fifth (relative to the fourth) than occurs in hands with negative findings.

The metacarpal sign was usually, though not invariably, found bilaterally in girls or women with gonadal dysgenesis (Table 3). It was present also in a few girls completely devoid of signs of gonadal dysgenesis and

TABLE 3. DISTRIBUTION OF METACARPAL SIGNS IN 17 PATIENTS WITH GONADAL DYSGENESIS. NONE OF THESE CASES HAS PREVIOUSLY BEEN REPORTED IN THE LITERATURE

Case No.	Nuclear chromatin bodies in cells of buccal smear	Metacarpal sign	Urinary gonadotropin (units/24 hrs.)	Chronologic age (yrs.)	Skeletal age (yrs.)	Height (ins.)	Avge. height for chron. age (ins.)
1*	neg.†	R+ L+	> 48	17	12.0	55	65
2	neg.	R+ L+	> 15	15	13.5	52	63
3	neg.	R+ L+	> 66	24	17.0	57	65
4	neg.†	R+ L+	> 53	16	11.5	49	64
5	neg.	R+ L+	>116	26	15.0	55	65
6	neg.	R+ L+	> 46	15	12.5	53	63
7	pos.	R+ L+	< 6	15	13.0	59	63
8	pos.	RB L+	—	13	11.5	50	60
9	pos.	RB L+	—	14	12.5	55	62
10	neg.†	R− L+	< 5	7	5.5	40	47
11	neg.	R+ L−	>212	19	14.5	56	65
12	pos.†	LB R−	>140	24	17.0	57	65
13	neg.	R− L−‡	> 20	17	15.0	52	65
14	pos.	RB LB	> 23	20	17.0	51	65
15	neg.†	RB LB	—	6	5.5	41	44
16	neg.†	R− L−	—	6	4.0	43	44
17	neg.†	R− L−	—	4	5.5	42	39

* See Figure 3.

† Sex chromatin status verified by examination of smears of "neutrophil enriched" blood.

‡ This patient had abnormally short fifth metacarpals in both hands (see Fig. 4).

with a normal menstrual history, and in a few women who had borne children. In at least some of these, the sign appeared to be a familial characteristic. In one family the metacarpal sign was found in a mother and 2 of her 3 daughters; all 4 were considered endocrinologically normal. There were 70 females in whom the metacarpal sign was either present or borderline in one or both hands. Buccal smears were obtained from 58 of these females; in 11 the nuclear chromatin pattern indicated the male sex. Among the 519 females with no metacarpal sign in either hand, buccal smears were obtained from 171; the sex chromatin pattern was female in all but 3 of these.

With remarkable frequency the metacarpal sign in males was associated with testicular atrophy, delayed sexual maturation or delayed testicular descent. However, none of our 5 cases of Klinefelter's syndrome (all with female sex chromatin) exhibited either the metacarpal sign or the borderline condition.

There were 241 males in whom the metacarpal sign was either definite or

borderline; in the 76 whose sex chromatin pattern was determined, it was found to be of the male type. There were 1754 males with no metacarpal sign in either hand; in 194 buccal smears were examined, and all but the 5 patients with Klinefelter's syndrome had male sex chromatin patterns.

Most children without the metacarpal sign continue thus through subsequent years. Nevertheless, as boys or girls get older, the tendency toward acquisition of the metacarpal sign increases; that is, in some cases the fourth metacarpal grows more slowly than the third or fifth. Of 26 whose metacarpal categories were observed to change during the period of observation, in 21 the findings became more positive; they moved from negative to borderline in 7, from negative to positive in 6, and from borderline to positive in 8. In only 5 cases did the findings change in the other direction—from borderline to negative in one or both hands; in these, the fourth metacarpal appeared to grow slightly faster than either the third or the fifth. In the few instances in which such a change from borderline to negative occurred, the degree of change was, on the average, much smaller than that observed when the change was in the other direction.

In 1 of our female patients (#13, Table 3) with gonadal dysgenesis and a male sex chromatin pattern, the metacarpal findings according to definition were negative, although there was marked shortening of the fifth metacarpals bilaterally (Fig. 4). Although this picture is reminiscent of that seen in pseudohypoparathyroidism (1, 2) or in pseudo-pseudohypoparathyroidism (3), the only other unusual attributes recognized as being shared by such patients and those with gonadal dysgenesis (Turner's syn-

FIG. 4

FIG. 4. Roentgenogram of left hand and wrist of a 16.5-year-old girl with gonadal dysgenesis (Case #13, Table 3). This illustrates an anomaly of the metacarpals found occasionally in gonadal dysgenesis, in pseudo-hypoparathyroidism and in pseudo-pseudo-hypoparathyroidism, but is not the metacarpal sign as defined in this article. The right hand had an equally short fifth metacarpal.

TABLE 4. CLASSIFICATION OF MALE PATIENTS ACCORDING TO DIAGNOSIS AND METACARPAL SIGNS

Diagnostic category	Metacarpal findings		
	Negative in both hands	Positive or borderline in one or both hands	
	Number	Number	Percentage for each diagnostic category
Gonadal anomalies			
Klinefelter's syndrome	5	0	0
Primary hypogonadism	0	1 (+1)*	100
Hypophyseal hypogonadism	2	4	67
Marked delay of sexual development; skeletal age within one year of chronologic age	80	14	15
Undescended testis:			
right	124	11	11
left		5	
Late spontaneous descent of one or both testes	135	33	20
Short stature for chronologic age			
Hypophyseal dwarfism	2	1	33
Hypothyroidism with epiphyseal dysgenesis	6	0	0
With >1 yr. delay in skeletal maturation	268 (+16)*	70 (+6)	31
With <1 yr. delay in skeletal maturation	63 (+ 4)	17	20
Achondroplasia	9	0	0
Other males			
Osgood-Schlatter disease	(+ 4)	(+3)	42
Epilepsy	1 (+ 4)	1 (+2)	37
Advanced maturation: prepuberal†	58	8	12
puberal and postpuberal†	66	12	15
Adrenocortical hyperplasia	1	0	0
Excessive height (beyond that expected on basis of advanced skeletal age)	15 (+20)	6 (+7)	27
Others (mostly siblings and other normal subjects)	919	58	5.9
Totals	1754	241	12.1

* Figures in parentheses indicate the number of patients already listed in another category and not included in totals at foot of table but included in calculations of percentages of positive findings within each category.

† Skeletal age more than two years ahead of chronologic age.

drome) are the short neck, and the tendency toward short stature and a round face. The levels of serum calcium and phosphorus were normal not only in Case 13 but also in all of the cases of gonadal dysgenesis tested, as well as in the cases of epilepsy listed in Table 4.

To determine the correlation between the presence of the metacarpal sign and the height, we recorded the ages, heights, average heights for age (based on data of Engelbach (4)), skeletal ages and the average heights for the skeletal ages for 15 randomly selected males who exhibited the metacarpal sign in both hands (Group 1, Table 5). Similar data were collected for 15 randomly selected males who did not have the metacarpal sign in either hand (Group 2, Table 5). The differences between measured height, and the heights expected for chronologic and for skeletal ages were calculated for the subjects in each group.

TABLE 5. RELATION OF SHORT STATURE AND DELAYED SKELETAL MATURATION TO METACARPAL SIGNS

	Group 1 (15 males with positive metacarpal findings in both hands)	Group 2 (15 males with negative metacarpal findings in both hands)
Range of chronologic ages (yrs.)	4–17	5–15
Range of skeletal ages (yrs.)	5–17	6–15
Mean chronologic age	13.1	11.7
Mean skeletal age	11.2	11.8
Mean of differences for each person		
Height less avge. height for chronologic age	−3.4	+0.2
Height less avge. height for skeletal age	−0.8	−0:07

It can be seen from Table 5 that the tendency for patients with the metacarpal sign to be of shorter than average stature for their chronologic age was quite striking. Because the average height of children has been increasing (5) since the Engelbach's standards (4) were published, the differences would be even more striking if modern standards were used. This discrepancy in height is still obvious, though less marked, when comparison is made with the average height expected on the basis of the skeletal age, as determined by the standards of Flory (6). Though the selection of subjects for the 2 groups was random, there was remarkably good correspondence between them as regards 1) the range of age and 2) the distribution of ages, and 3) especially the distribution of skeletal ages. It is apparent from examination of Table 5 that persons with the metacarpal sign tend to show an appreciable delay in skeletal maturation.

DISCUSSION

The presence of short metacarpals has been mentioned previously in the literature in single case histories of patients with gonadal dysgenesis (7–10). In some cases it has passed without comment, though apparent in the roentgenograms (11, 12). However, the frequency of this finding in gonadal dysgenesis and in the population in general and the possible significance of the finding in males have not been determined heretofore. A positive (13–17) or borderline (18, 19) metacarpal finding is evident in a number of published roentgenograms, in melorheostosis (20, 21), and in at least 1 case of ectodermal chondrodysplasia (22) although it is unusual in this condition. The metacarpal sign was not present in our 3 cases of osteogenesis imperfecta, 12 cases of achondroplastic dwarfism, and 1 case of Hurler's syndrome. Three of our 4 cases of Morquio-Brailsford's type of chondrodysplasia did not show the metacarpal sign.

When the metacarpal sign is detectable in more than one generation of the same family, no endocrine significance is attached to this finding. When, however, it occurs in only 1 member, the chances of there being some aberration of the gonads are very high. This aberration may be only a late descent of one or both testes (*cf.* (17), Case 1), which may later develop to presumably adequate size and normal function. Although there were many males with cryptorchism and no metacarpal sign in either hand, the incidence of the metacarpal sign in males with late spontaneous descent of the testes was more than double the incidence in the normal boys of our group. Although the proportion of apparently sterile males is much higher among those with positive metacarpal findings than among the population in general, a positive finding is by no means incompatible with fertility. Indeed it is believed that most of the male patients exhibiting the metacarpal sign either are, or will be fertile.

The mechanisms which control the development of the relative length of the metacarpals are not recognized (23). Because of the many skeletal and other congenital anomalies found in patients with gonadal dysgenesis, it might be assumed that the metacarpal sign is merely another skeletal anomaly. However, the occurrence of the sign in males with marked gonadal involvement is not associated with any congenital skeletal abnormalities which have yet been recognized. The work of Pol, as reported through Beard by Albright and Reifenstein (1, p. 44), attempts to account for the relative lengths of the metacarpals. Those metacarpals which are prone to be shortest are said to acquire their epiphyses and begin cartilage proliferation latest. The details of the original work by Pol are not given and other references to this work in the literature, though sought by us, have not been discovered. It appears that his only publication in this field (23) is an extensive evaluation of the hypotheses of others.

The fact that the metacarpal sign is found more frequently in the left hand than in the right and that positive findings tend to be *more* strongly positive, and negative findings are *more* nearly borderline on the left than on the right, might be thought to be due to the greater frequency of right-hand dominance. If this were the case, the dominant hand would tend to yield only negative findings. Only a few of the subjects whose right hand alone showed the metacarpal sign were investigated in this connection. Of these, none was left-handed. Hence, hand dominance does not appear to be the factor which determines the metacarpal sign.

A high incidence of positive metacarpal findings was observed by us in a very small series of patients with rheumatoid arthritis not included in the present series (*cf.* also 18). Unfortunately, the population of patients available to us is not suitable for adequate evaluation of the incidence of positive metacarpal findings either in rheumatoid arthritis or in the un-

related condition, sterility. Nevertheless, on the basis of our findings in gonadal dysgenesis and hypophyseal and primary hypogonadism, it is believed that the incidence of the metacarpal sign in cases of sterility would be high.

Because of the high incidence of positive metacarpal findings in the few epileptics of our series and the tendency to short metacarpal formation in pseudohypoparathyroidism, it would be well to compare the findings in these disorders in which convulsions or tetany occur even though the epileptic patients had normal levels of serum calcium and phosphorus at the time their blood was sampled.

The data presented in this study do not necessarily represent those typical of the general population, for the group here analyzed has a lower mean age, a shorter mean stature (even for any specific age group), a greater proportion of males, and a higher incidence of gonadal anomalies than the general population. Moreover, short persons, those with delayed maturation and those with gonadal aberrations are more likely than most persons to seek help in endocrine and growth-study clinics. The incidence of positive metacarpal findings would be expected to be much lower in the general population.

Acknowledgment

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Addendum

After this manuscript was prepared, the paper by Ten Bosch (24) came to our attention. Of 8 patients with very short metacarpals, 6 were phenotypic females and of 4 tested, 3 had male sex chromatin. Serum calcium and phosphorus levels were normal in all 8 patients, though the oldest at age 49 had calcinosis. Apparently some of these patients and one of our patients (Case 13, Table 3 and Fig. 4) meet the requirements for a diagnosis of both gonadal dysgenesis and pseudo-pseudohypoparathyroidism. However, Ten Bosch's paper is concerned with extremely short metacarpals rather than the slight shortness of the fourth metacarpals relative to the third and the fifth. Hence, his statement that "a study of hands of 25 other cases of gonadal dysgenesis failed to show abnormal metacarpal bones" is consistent with the high incidence (82 per cent) of positive metacarpal findings reported in Table 3.

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A CASE STUDY OF PECULIAR SOFT-TISSUE AND BONY CHANGES IN ASSOCIATION WITH THYROID DISEASE

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ABSTRACT

A case of thyroid acropachy is described. The pertinent literature is reviewed and this case discussed in terms of the other reports. Thyroid acropachy refers to a peculiar periosteal proliferation of bone limited to the metacarpals, metatarsals and phalanges, associated with terminal phalangeal clubbing. The patient first showed these bony changes following radioactive iodine therapy for Graves' disease at a time when she was considered euthyroid. In association with the bony changes there was swelling of the soft tissues overlying the involved phalanges and also unusually marked swelling of the orbital soft tissues. The pathology of the bony and soft-tissue lesions is described; it is not compatible with a diagnosis of either systemic or localized myxedema. The anterior pituitary gland and its secretory products probably play an important etiologic role in this condition.

IN THE mind of the clinician, clubbing of the digits is commonly associated with pulmonary disease and occasionally with cardiac or gastroenteric disturbances; rarely, it may be unassociated with any other pathologic condition. However, it may also be associated with thyroid disease—so-called thyroid acropachy (1). The basis for this relationship is no more understood than is the relationship between digital clubbing and pulmonary disorders. The following report concerns a patient with thyroid acropachy who has been closely followed prior to, during, and after the onset of the disorder.

CASE REPORT

D.M., a 45-year-old single colored woman, was admitted to the Orthopedic Service of the Cincinnati General Hospital on May 22, 1954. She had sustained a compression fracture of the first lumbar vertebra as a result of a fall. The history indicated easy fatigability, slight dyspnea on exertion, excessive sweating, recent onset of prominence of the eyes and a 40-pound weight loss during the previous year despite an increasing appetite. In addition, she complained of nervousness which had been present ever since

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FIG. 1. Patient D. M. in December 1955. Note the swelling of the orbital soft tissues.

she had undergone a hysterectomy for uterine fibroids in 1947 but which had become worse in the previous year. She was known to have syphilis and hypertension.

Physical examination on admission revealed a well-nourished, hyperactive, middle-aged colored female. The blood pressure was 150/100 mm. Hg. The pulse was that of auricular fibrillation, with a rate of 144 per minute. The eyes exhibited stare and there was minimal supraglobar swelling of the soft tissues. The skin was warm and smooth, with scanty growth of hair. There was a soft, smooth, and diffusely enlarged thyroid, the estimated weight of which was 50 grams. Visible precordial pulsations were present and the heart sounds were loud. She displayed a fine tremor of her fingers.

The urinalysis findings, a complete blood count, and the blood levels of creatinine, urea nitrogen, calcium, phosphorus, cholesterol and protein were normal. The serologic test for syphilis gave a reading of 2 plus. Thyroid function studies demonstrated a basal metabolic rate of +60 per cent and a thyroidal radioactive iodine uptake of 71 per cent in twenty-four hours.

While a decision regarding definitive therapy was pending the patient received 30 mg. of Tapazole and 65 mg. of phenobarbital daily, in fractional doses. The dosage of Tapazole was increased gradually to 80 mg. daily. When it was discontinued on July 1, the patient still had tachycardia (sinus rhythm), and still manifested evidence of thyrotoxicosis although the nervousness and hyperactivity had improved. On July 8, 1954,

she was given 7.5 millicuries of radioactive iodine (I^{131}) by mouth.

Two months after treatment with I^{131} the patient was considered to be probably euthyroid. Her eyes demonstrated increasing swelling of the periglobar and upper-lid soft tissues. The 24-hour thyroidal I^{131} uptake, determined shortly thereafter, was 28 per cent and the basal metabolic rate was +4 per cent. In November 1954, the orbital soft-tissue swelling seemed to have increased further and in March 1955, she said that her eyes were watering more. Because of this progressive orbital congestion and because of recurrent nervousness, failure to gain weight, continued tachycardia, a rise of the basal metabolic rate to +65 per cent, and an increase of the 24-hour thyroidal I^{131} uptake to 65 per cent, she was considered to be thyrotoxic again. She was given 11.2 millicuries of I^{131} on March 10, 1955 and rapidly lost all symptoms and signs of hyperthyroidism. The orbital and eyelid puffiness became considerably worse and was soon associated with increased pigmentation of the orbital skin and moderate vascular injection of the bulbar conjectiva. In addition, swelling of the ankles and pretibial soft tissue developed. Treatment with digitalis, ammonium chloride and Mercuhydrin resulted in improvement only of the dependant edema.

At various times between April 1955 and early 1957 the patient underwent prolonged courses of therapy, singly and in various combinations, with adequate oral doses of desiccated thyroid, triiodothyronine, cortisone and prednisone, in an effort to relieve the swelling of the orbital soft tissues. Cortisone in the form of an ophthalmic ointment was even applied locally. Although the amount of orbital swelling varied slightly from time to time there was no correlation between the improvement and the use of a particular therapeutic agent.

In December 1955, the periorbital swelling was still prominent. Luedde exophthalmometer readings were 27 mm. (OS) and 26 mm. (OD) (Fig. 1), and there was a moderate degree of chemosis. Bilateral temporal myopic cones were manifest on funduscopic examination and the proptosis, though considerable, was easily reducible.

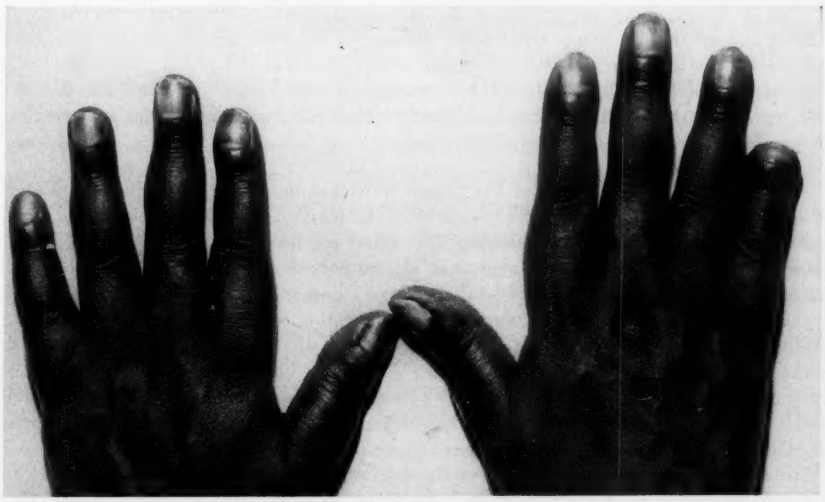

FIG. 2. Hands of Patient D. M. in July 1956. The unrelated disfigurement of the fifth digit on the right hand was a result of trauma. Note the swelling of the soft tissues and the clubbing of the terminal phalanges.

FIG. 3. Roentgenograms of Patient D. M.'s hands in July 1956. **a**, The whole hand The apparent fracture line in the middle phalanx of the fifth digit is an artefact. Note the marked periosteal reaction involving all of the proximal and middle phalanges; this process was bilaterally symmetrical. **b**, A magnified view of the periosteal reaction involving one of the proximal phalanges seen in (**a**). Note that the spicules are oriented perpendicularly to the long axes of the phalanx.

In July 1956, abnormalities of her fingers were first noted (Fig. 2). These consisted of symmetrical swelling of the soft tissues of all the midlle and proximal phalanges without alteration in texture of the overlying skin. There was definite clubbing of the terminal phalanges. The swelling of the fingers was at first soft in consistency, but later felt indurated. Roentgenograms (Fig. 3), taken in the summer of 1956, showed a periosteal reaction limited to the bony phalanges underlying the swelling of the soft tissues. In addition to subperiosteal formation of new bone of the ordinary variety, there was cortical thickening of fairly severe degree; spicules of bone projected at right angles to the long axes of some of the affected phalanges. The metacarpals were unaffected and there was no evidence of periostitis or other abnormality in any of the other bones of the body, including the orbital bones.

Neither the periorbital swelling nor the swelling of the fingers responded to any of the various types of therapy described. In early 1958 the patient first demonstrated clinical evidence of hypothyroidism. The basal metabolic rate at that time was only −5 per cent but the concentration of serum protein-bound iodine had dropped from 4.0 μg. (summer of 1956) to 2.6 μg. per 100 ml. The 24-hour thyroidal I^{131} uptake remained

at approximately 10 per cent, the value noted in the summer of 1956. Oral administration of desiccated thyroid was started and was followed by the general improvement expected in myxedematous patients, which persisted during therapy with 200 mg. of thyroid daily. No alterations of the abnormality of orbital soft tissues has been noted to date. On the other hand, the swelling of the digital soft tissues gradually diminished during 1958, after the patient started thyroid, and was barely discernible by May 1959.

Pathology report. Biopsy specimens were obtained in 1957 of skin from one of the affected digits and of bone from the same digit. Sections of the specimen of bone revealed only minute fragments of cortex in close proximity to the periosteum. The latter showed a peculiar nodular fibrosis and in the subjacent bone there was subperiosteal formation of new bone, with fibrosis of the intervening marrow spaces. No inflammatory reaction was noted. The subperiosteal formation of bone resembled that seen in hypertrophic osteoarthropathy, but nodular fibrosis of the periosteum is not usually a feature of that disease. The fragments were too minute for further interpretation. Staining of sections of the skin with toluidine blue revealed metachromasia confined to scattered mast cells in the soft tissue. The skin was otherwise normal.

A biopsy was made of this patient's orbital contents in 1958; the sections of skin and orbital fibroadipose and muscular tissue obtained showed only mild separation of the muscle fibers, which was of questionable significance. Toluidine blue staining again showed metachromasia only in mast cells. Alcian blue staining revealed material digestible by bacterial hyaluronidase between fibers. Sulfation followed by staining with toluidine blue showed that there was no metachromasia between fibers. Although acid mucopolysaccharide (probably hyaluronic acid) was presumably present, the findings described do not warrant the conclusion that it was increased, as in myxedema. There was nothing to suggest the diagnosis of "localized myxedema" of the skin.

DISCUSSION

Data on the documented cases of thyroid acropachy in the literature are presented in Table 1. The roentgenographic findings in our case were typical of what has been found in 7 of 8 cases of thyroid acropachy reported in the literature. Although the clubbing observed in this patient was similar to that seen in patients with hypertrophic osteoarthropathy, the type of periosteal reaction with spicule formation oriented perpendicularly to the long axis of the bones involved, the location of this reaction away from the ends of bones, and the peculiar limitation of the reaction to the phalanges rather than the larger bones, were features apparently seen only in association with thyroid disease. Acromegaly may cause clubbing of the terminal phalanges and might have been a factor in Greene's case (4), but the periosteal reaction noted in our patient (D.M.) is not a feature of acromegaly. Patient D.M. had inadequately treated syphilis and a positive serologic reaction to the test for syphilis at the time of investigation, thus raising the question of syphilitic periostitis. This diagnosis can be dismissed, however, because of the location and symmetry of the periosteal lesion.

The patient described here is apparently the thirteenth instance of thyroid acropachy to be reported (Table 1). Eleven of the patients demonstrated obvious spontaneous hyperthyroidism at some time, and appar-

TABLE 1. DOCUMENTED CASES OF THYROID ACROPACHY IN THE LITERATURE

Author	Exophthalmos	Pretibial myxedema	Digital clubbing	Soft-tissue swelling of fingers	Periosteal reaction	Hypothyroidism	Previous therapy	Time of onset of clubbing and/or periosteal reaction	Hyperthyroidism	Age of patient when first seen	Comments
Thomas (1)	Yes	Yes	Yes	Yes	Yes	Yes	Operation on thyroid	8 months after operation	Yes	22 yrs. (male)	First seen when hyperthyroid, before therapy. Exophthalmos preceded therapy
Cushing (2)	Yes	Yes	Yes	Not clear in report	Not clear in report	Not clear in report	Operation on thyroid	8–9 years after operation	Yes	28 yrs. (male)	First seen when hyperthyroid, before therapy
Rynearson and Sacasa (3)	Yes	Yes	Yes	No	Yes	Yes	Operation on thyroid	6 weeks after operation	Yes	43 yrs. (male)	First seen when euthyroid, 2 years after therapy. Exophthalmos already present
Greene (4)	Yes (severe)	Yes	Yes	Yes	Yes	Not clear in report	Iodides	Present when first seen, at age 74	Not clear in report	74 yrs. (male)	First seen when euthyroid, 18 years after therapy. Clubbing and exophthalmos probably were present prior to therapy. McGill reports that this case later had additional diagnosis of eosinophilic adenoma of pituitary
Engel (5)	Yes	Yes	Yes	Yes	Yes	Yes	Iodides; propylthiouracil; operation on thyroid; pituitary irradiation	1 year after operation	Not clear in report	41 yrs. (female)	First seen when euthyroid, 4 months after therapy for what may not have been spontaneous hyperthyroidism. Exophthalmos preceded surgery; surgery and pituitary irradiation were followed by systemic myxedema; clubbing followed myxedema
Inch and Rolland (6) (Case 1)	Yes	Yes	Yes	No	No x-ray exam.	Yes	Methyl thiouracil; operation on thyroid	9 months after operation	Yes	36 yrs. (female)	First seen when hyperthyroid, before therapy. Exophthalmos preceded operation; pretibial myxedema and clubbing followed operation
(Case 2)	Yes (severe)	Yes	Yes	No	No x-ray exam.	No	External thyroid irradiation	19 years after irradiation therapy	Yes	39 yrs. (male)	First seen when euthyroid, 19 years after therapy. Exophthalmos developed soon after irradiation; pretibial myxedema developed 16 years after irradiation
Warthin and Boshell (7)	Yes	Yes	Yes	No	No x-ray exam.	No	Operation on thyroid	2 years after operation	Yes	29 yrs. (male)	First seen when euthyroid, 2 years after therapy. Pretibial myxedema present
McGill (8)	Yes	Yes	Yes	No	Yes	No	Thiourea; operation on thyroid	2 years after operation	Yes	51 yrs. (male)	First seen when hyperthyroid, before therapy. Exophthalmos followed therapy and preceded clubbing. Nodular goiter removed at operation
Freeman (9) Case 1)	Yes (severe)	No	Yes	No	Yes	Yes	Operation on thyroid; external thyroid irradiation	Present when first seen	Yes	56 yrs. (male)	First seen when euthyroid, 28 years after therapy. Pulmonary emphysema present also
(Case 2)	Yes (severe)	No	Yes	No	No	No	Radioiodine	Present when first seen	Yes	54 yrs. (male)	First seen when hyperthyroid, before therapy
Danforth and Humphrey (10)	Yes	Yes	Yes	Yes	Yes	No	Radioiodine	Within 10 months of radioiodine	Yes	46 yrs. (male)	First seen when euthyroid, 5 years after therapy
King *et al.*	Yes	No	Yes	Yes	Yes	Yes	Tapazole; radioiodine	2 years after radioiodine	Yes	45 yrs. (female)	Present case (D.M.)

ently it was the classic form of hyperthyroidism (Graves' disease) in most instances. Unfortunately the clinical features and pathologic description of the goiters were not always recorded. The description of Greene's patient is too incomplete to permit a decision regarding the existence of hyperthyroidism, and in Engel's patient the hyperthyroidism may have been drug-induced.

It should be noted that 10 of the 13 patients were males, yet the incidence of hyperthyroidism, and in particular that of Graves' disease, is four or five times as common among females as males. The significance of this reversal of sex incidence requires further investigation. All 13 patients demonstrated clubbing of digits. In 10 of the 11 patients with spontaneous hyperthyroidism the digital clubbing first developed after subsidence of the manifestations of hyperthyroidism; in 1 patient both conditions developed simultaneously. Greene's patient exhibited digital clubbing before any therapy for thyroid disease was instituted, whereas Engel's patient received antithyroid therapy before digital clubbing appeared. However, the time-interval between therapy for hyperthyroidism and the onset of digital alterations varied considerably in different patients and often was not carefully documented. Exophthalmos,[1] eventually manifest in all 13 patients, was present prior to therapy in 11; it progressed after therapy for hyperthyroidism in 9.

In 6 of the 13 patients, systemic myxedema was eventually recognized. In 2 of the 13 too few data were available to permit appraisal. In only 1 of the 6 cases with systemic myxedema did manifestations of this condition appear before clubbing was noted (5). In the present case, the development of clubbing and of periosteal new bone followed effective treatment of the hyperthyroidism, but definitely preceded any evidence of systemic myxedema. Moreover, the digital lesions did not change as the patient became myxedematous. Treatment with desiccated thyroid, although it caused improvement of the systemic myxedema and possibly of the digital swelling, did not improve the abnormalities of the bones, orbital tissue, or the eyes. Ten of the 12 previously reported cases of thyroid acropachy manifested well defined pretibial myxedema. In the present case the appearance of the skin overlying the phalanges and the periorbital soft tissue, did not resemble that seen in localized myxedema, either clinically or histologically.

The existence of exophthalmos and of localized pretibial myxedema suggests some type of anterior pituitary oversecretion as the cause of the changes seen in this patient's digital and orbital tissues. The concentration

[1] The term exophthalmos is arbitrarily used to include all disorders of the eyeball and orbital tissue believed to be associated with hyperfunction of the thyroid or anterior pituitary.

of serum thyroid-stimulating hormone (TSH) has been found to be elevated (bioassay method of Gilliland and Strudwick (11)) in 2 cases of thyroid acropachy. Nevertheless, this does not indicate a pituitary relationship, since the same investigators found an elevated serum level of TSH in all of their cases of thyrotoxicosis with progressive exophthalmos and in nearly all patients with spontaneous or induced myxedema.

Physiologically, clubbing of the phalanges has been attributed to local hypoxia. One author (1) suggested that this need be only a relative hypoxia, such as would be anticipated in a sudden change from the hyperthyroid to the euthyroid or hypothyroid state. No such situation can be substantiated in our patient. The reports of several investigators implicate other local tissue factors. Changes in digital blood flow have been found in patients with hypertrophic pulmonary osteoarthropathy (12), and there has been regression of clubbing following unilateral vagal section (13). Apparently some local tissue factor plays a critical role in the genesis of the condition described, since these phalangeal and orbital changes occur only rarely in the course of an extremely common disease.

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TRANSIENT RECURRENT GYNECOMASTIA AFTER REMOVAL OF AN ESTROGEN-SECRETING INTERSTITIAL-CELL TUMOR OF THE TESTIS*

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ABSTRACT

A young man with an interstitial-cell tumor of the left testis had bilateral gynecomastia, atrophy of the right testis, and changes in the skin, beard, and voice suggesting decreased androgenic effects. Fertility, normal libido, and potency were retained. The excretion of estrogen was elevated, that of gonadotropin was depressed and that of 17-ketosteroids was normal. In the extratumoral area of the left testis, spermatogenesis was somewhat decreased, there was moderate hypoplasia of the germinal cells, and interstitial cells were sparse. These findings are attributed to secretion of moderate amounts of estrogen by the tumor and resultant suppression of pituitary gonadotropin secretion. After left orchiectomy, the gynecomastia and hypoandrogenic effects subsided rapidly and completely. One week postoperatively 17-ketosteroid excretion had doubled. The right testis had increased to normal size and consistency by eight months after operation. Gynecomastia recurred unilaterally four months postoperatively, was noted to be bilateral but moderate in degree nine and eleven months later, and disappeared again by twenty-two months after removal of the tumor. After surgery, gonadotropin excretion was at the upper limit of normal for many months, 17-ketosteroid excretion remained in the high-normal range, and estrogen excretion declined slowly to normal. This postoperative course is attributed primarily to the "rebound" of pituitary gonadotropin output following excision of the estrogen-producing tumor. Mechanisms underlying the recurrent gynecomastia are discussed.

INTERSTITIAL-cell tumor of the testis occurs rarely in humans (1–4). Prepubertal and postpubertal cases comprise two distinct clinical entities. All prepubertal patients (5–18) have displayed precocious virilization, with the uninvolved testis remaining small. High 17-ketosteroid excretion for the child's age has been found in all of the cases studied (5, 9–

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18, 19–23). Definite gynecomastia has been seen in only 3 children (8, 18, 24); the only hormonal findings were negative titers for chorionic gonadotropin in 2 of them (8, 24).

In contrast, the majority of adult patients have shown no endocrine disturbances, but gynecomastia has been reported in about 30 per cent (3, 4, 25–39).[1] Gynecomastia has subsided after excision of the tumor in all but 2 cases (29, 37). In most of the cases with sufficient laboratory determinations, excessive estrogen has been found either in the tumor (26, 28) or in the urine (28, 30, 31, 36–39). Reduction of estrogen excretion to normal after orchiectomy has also been noted in cases studied postoperatively (30, 31, 36, 38). Effects on the function or morphology of the extra-tumoral testicular tissue, presumably secondary to the known ability of estrogen to suppress secretion of pituitary gonadotropin (40–42), have also been reported in a number of these cases. Depression of the seminiferous tubules has been evident in atrophy of the contralateral testis (30, 31, 38) or in tubular degeneration and impaired spermatogenesis (26, 29, 31, 34, 37, 39).[2] Depression of the interstitial cells has been manifested in a decrease of libido and potency (26, 27, 31, 33, 34, 38, 39),[2] of male secondary sexual characteristics (31), of secretion of total or testicular androgens (30, 36, 39), or of the number of interstitial cells (34, 37, 39).[2]

However, in 1 probable (32) and 2 definite (4, 35) cases of interstitial-cell tumor in adults with gynecomastia, the excretion of estrogen has been reported to be normal; in 2 of them (32, 35) pregnanediol excretion was elevated. Urinary androgen (by bioassay) or 17-ketosteroid excretion has been determined in a number of adult patients with gynecomastia, and has proved to be normal or low in all (4, 30–32, 35–38, 39). In contrast, Masson (43) and Venning (44) have reported an adult with markedly elevated 17-ketosteroid excretion due to a widely metastatic interstitial-cell tumor; no endocrinopathy was noted clinically.

In previous cases, observations on the course of recovery after operation have been limited. The present case affords correlation of repeated clinical and laboratory studies in a young man with an estrogen-secreting interstitial-cell tumor. Before operation, gynecomastia and partial testicular depression were associated with elevated excretion of estrogen and de-

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[1] The cases in references 30, 31 and 32 are included as probable examples of interstitial-cell tumor, although an unequivocal diagnosis was not reached by the authors. The diagnosis of interstitial-cell tumor in the case reported in reference 3 is doubtful. The reasons will be discussed fully in a subsequent review article.

[2] Estrogen determinations were not obtained in the cases in references 29, 33 and 34. Histologic changes in the non-tumorous testicular tissue were not mentioned in the reports, but in 2 cases (29, 34) were found here on review of the slides, generously loaned by the authors.

pressed excretion of gonadotropin; postoperatively, the original abnormalities subsided and the picture of a "second puberty," including transient recurrence of gynecomastia, developed.

CASE REPORT

D.F.B., a 23-year-old white man, entered the Seattle Veterans Administration Hospital in January 1957, complaining of bilateral breast enlargement. Four years prior to admission (p.t.a.) he had noted tenderness and then gradual enlargement of both breasts. A plastic breast operation was performed bilaterally. Three years p.t.a. he had first noted a lump in the left testis. One year p.t.a., bilateral tenderness and enlargement of the breasts had returned; the patient thought it probable that insidious enlargement of the left breast had begun three years p.t.a. He had shaved only every two to three days for several years p.t.a. He felt healthy in every other respect. Libido and potency were normal and unchanged. His first child was born one year p.t.a.

Physical examination revealed a husky young man with fairly soft, smooth skin, a light beard, moderate bilateral gynecomastia (more on the left than on the right), an atrophic right testis (half the size of the left), and a slightly enlarged left testis with a firm nodule making up the lower two-thirds. Other findings were normal.

Laboratory findings. An intravenous pyelogram and chest roentgenogram were not remarkable. A Friedman (serum chorionic gonadotropin) test yielded negative results. Urinary hormonal determinations are recorded in Table 1. Estrogen was elevated and gonadotropin was not detected (<3 units; normal range, 6–48 units).

Course. On January 7, 1957, the patient underwent left orchiectomy; a local tumor was found to have replaced most of the left testis. There was no evidence of spread. The pathology report was as follows: Gross: The testis measured $4.0\times3.5\times1.8$ cm. The external surface exhibited a smooth tunica albuginea with slightly congested vessels near the inferior pole. On the cut surface of the testis there was a bulging, well-circumscribed neoplasm measuring approximately $2.2\times2.5\times2.0$ cm. The cut surface appeared variegated, with irregular islands of soft yellow or brownish-tan tissue with small focal areas of congestion and softening. Near the border of the neoplasm there were greyish-white strands of fibrous connective tissue, which in one area appeared to be adherent to the overlying tunica albuginea. The adjacent testis presented the usual yellowish, homogeneous cut surface of the normal gland. No remarkable features were noted about the attached epididymis and cord. Mutliple representative sections were taken. Microscopic: Histologic examination of the left testis, containing the neoplasm, showed an interstitial-cell tumor arising within the gland. The tumor for the most part consisted of well-differentiated interstitial cells (Leydig cells) having a prominent oval to round nucleus, relatively abundant eosinophilic cytoplasm, and a definite border. In some areas they were rather closely packed and showed uniformity throughout. Relatively rare mitoses were noted. The nuclei were hypochromatic. There was a well defined capsule separating the tumor from the adjacent compressed testis. Prominent fibrous trabeculi with well defined blood vessels were interspersed throughout the tumor. In these areas the tumor cells appeared to be less dense. Some cells showed vacuolization of the cytoplasm. No definite crystalloids were identified. In the spermatic tubules of the adjacent testis there was decreased but active spermatogenesis, with moderate hypoplasia of the germinal epithelium. The interstitial cells in the adjacent testis were relatively sparse. Sections stained with Oil-Red-O showed abundant lipid material within the cytoplasm of the tumor cells.

No biopsy specimen was obtained from the right testis.

TABLE 1. URINARY HORMONE DETERMINATIONS

Date	Gonadotropin (mouse units/24 hrs.)	Estrogen (rat units/24 hrs.)	17-Ketosteroids (mg./24 hrs.)	17-Hydroxycorticoids (mg./24 hrs.)
1957 Jan. 5	<3 (− at 3)	30 (− at 40)	17.5	7.8
Left orchiectomy Jan. 7, 1957				
Jan. 14			32.2	4.7
Feb. 27	≥6 (higher dilutions not tested)	≥24 (higher dilutions not tested)	26.7	5.4
May 22	48 (− at 96)	≤30 (− at 40)*	22.5	1.1
Sept. 10	96 (− at 192)	<10 (− at 10)	10.1	10.1
Oct. 24	48 (− at 96)	≤18 (− at 24)*		
1958 Feb. 13			24.6	12.6
Apr. 21		10 (− at 15)		
Nov. 7	24 (− at 48)	10 (− at 15)	21.8	12.6

* The overnight (10:00 P.M.–5:00 A.M.) specimen was mistakenly included in addition to the true 24-hour specimen. In the absence of information on diurnal variation in estrogen production, the correct 24-hour value could be 50 to 100 per cent of the value listed.

Figures 1, 2 and 3 show the microscopic morphology of the tumor and the remainder of the left testis.

Within a few days after surgery, the gynecomastia had regressed markedly, the beard had become heavier and darker, and the voice had become lower. One week postoperatively, urinary 17-ketosteroid excretion had doubled. The patient gained 25 pounds within three weeks, and his family independently noted a darker beard and a deeper voice. Seven weeks after operation he reported increased strength and stamina, although they had seemed normal previously; he was shaving once or twice daily; there was no gynecomastia. Four and a half months postoperatively he complained of mild fatigue and tenderness of the left breast, present for five weeks; there was a deep subareolar flat disc, 1½ cm. in diameter, in the left breast; a month later the condition was unchanged. Seven and a half months after operation the patient was re-examined because he felt that his remaining testis had enlarged rapidly in the previous few weeks; the findings were normal except for slight unilateral gynecomastia, as on the last visit, and absence of

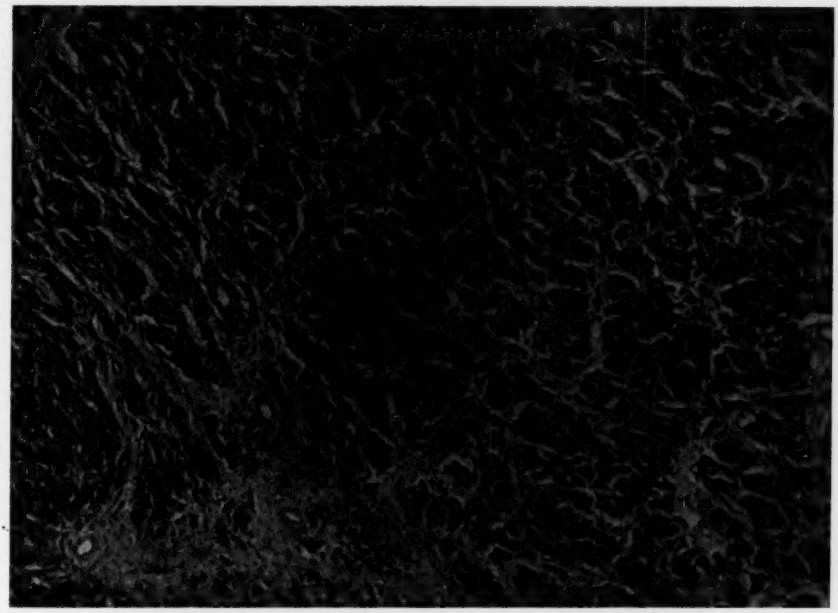

FIG. 1. Section of the interstitial-cell tumor (hematoxylin and eosin stain, ×243.)

the left testis; the right testis now measured 3.5×5.5×2.5 cm. and was of normal shape and consistency. Eight months postoperatively, the results of examination were similar; review of the records confirmed that the right testis had gradually grown to normal size since operation; a roentgenogram showed the chest to be virtually unchanged. A month later his second child was born. On examination nine and a half months after operation, there was still the same "button" of tissue in the left breast. Thirteen months postoperatively there was slight but definite bilateral gynecomastia, 1–2 cm. in diameter, more on the left than on the right. Two and a half months later the mammary condition was similar; other physical findings and the chest roentgenogram remained unchange Twenty-two months after operation the gynecomastia had subsided completely on the right and almost completely on the left, where there was some question of a ½-cm. nodule; the rest of the physical findings were again normal, except for absence of the left testis.

Table 1 shows the results of all hormone assays during the postoperative course. After surgery, urinary gonadotropin titers were at the upper limits of normal but ultimately tended to decline; 17-ketosteroid excretion rose promptly and remained in the high normal range (except for the observation at eight months postoperatively); and estrogen excretion subsided slowly to normal.

ASSAY METHODS

Determinations of gonadotropin in serum (Friedman test) and urine were performed by the Laboratory of Clinical Medicine, Seattle. The urinary assay utilized the uterine-weight response of immature (21-day-old) mice (45) tested with urine processed by

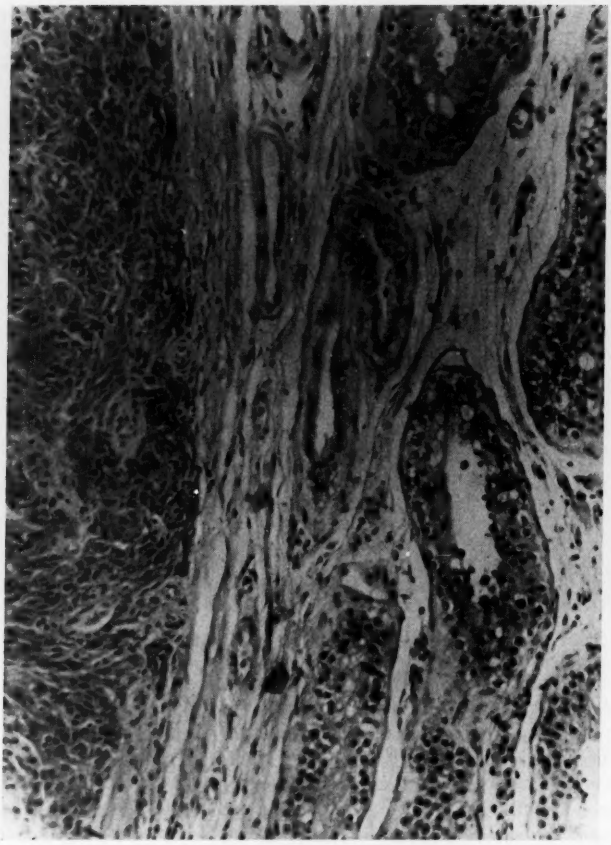

FIG. 2. Junction of the interstitial-cell tumor and adjacent testis (hematoxylin and eosin stain, ×238).

adsorption of gonadotropin(s) on kaolin according to the method of Bradbury, Brown and Brown (46). The normal range of urinary gonadotropin is 6–48 units, in agreement with the experience of Klinefelter *et al.* (47). Estrogen assay was carried out by Dr. Elizabeth K. Smith, formerly of the University of Washington Department of Obstetrics and Gynecology, using vaginal cornification in the ovariectomized rat in response to the injection of the phenolic fraction of the urinary steroid extract. A priming dose of 2 μg. of estrone was given to each rat one week before the assay, and only those responding to this dose with positive findings in the vaginal smear were used in the final assay. The normal range of estrogen excretion is 6–50 units in cycling women; in 2 normal young men, it was 10 units (not detectable at 15 units). A concurrent standard was not used in either the estrogen or gonadotropin assay (48). Duplicate estrogen determinations, carried out in different weeks, usually gave the same result and never differed beyond one dilution.

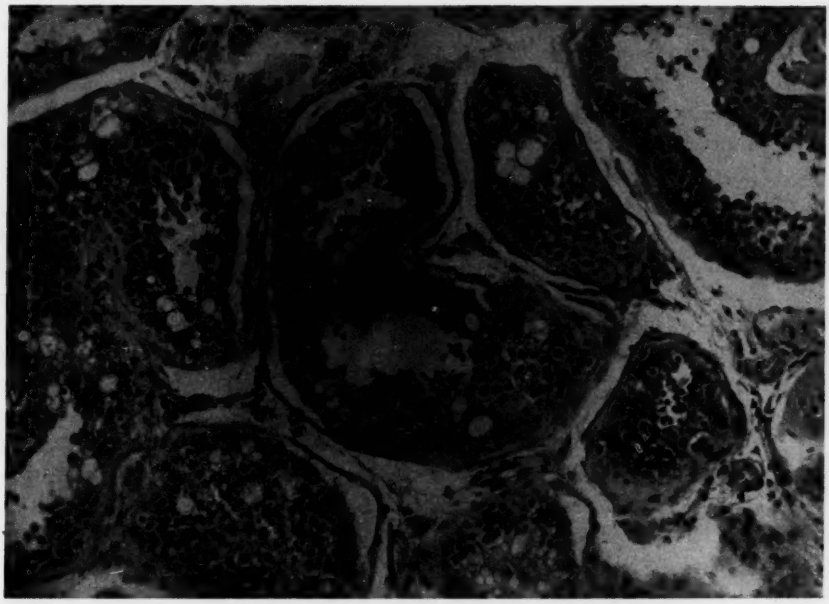

FIG. 3. Section of the extra-turmoral tissue of the involved testis (hematoxylin and eosin stain, ×347).

Urinary 17-ketosteroids (method of Drekter *et al.* (49) and 17-hydroxycorticoids (method of Brown (50)) were determined by the Seattle Veterans Administration Hospital Clinical Laboratory.

DISCUSSION

Tumor morphology. The gross and microscopic appearance of the tumor in this case was typical of interstitial-cell tumors (2, 29). These growths may or may not be encapsulated (2).

Endocrine changes due to the tumor. The tumor has been assayed in only 2 cases with gynecomastia, and the estrogen content has been high (26, 28). Secretion of estrogen by the tumor was evidently the primary cause of endocrinopathic manifestations in the present case. Since the excretion of estrogen was high preoperatively and ultimately subsided to normal after removal of the involved testis, the tumor (not a remote source) must have caused the excess of estrogen. The normal excretion of 17-hydroxycorticoids and 17-ketosteroids preoperatively militates against the theoretical possibility that the tumor secreted an ACTH-like substance, eliciting increased adrenal secretion of estrogen. The low excretion of gonadotropin and the normal excretion of 17-ketosteroids preoperatively indicate that

the elevation in estrogen output was not secondary to increased production of either gonadotropin or androgen.

Gynecomastia was, undoubtedly, a direct estrogenic effect, and estrogen suppression of pituitary secretion of gonadotropin was reflected by the low excretion of gonadotropin and secondary testicular effects. Estrogens have been shown to be more potent than testosterone in depressing the output of gonadotropin (48, 51, 52), and estrogen therapy has been shown to cause, through its action on the pituitary, both degeneration of the germinal epithelium and diminution or disappearance of the interstitial cells (40–42, 52). Thus the contralateral testicular atrophy was a manifestation of degeneration of the seminiferous tubules,[3] secondary to the depression of the gonadotropin level. The subnormal growth of the beard and the higher voice could be attributed both to antagonism by estrogen of androgenic effects on end-organs (53, p. 133) and to the decreased output of testosterone by extra-tumoral interstitial cells, secondary to the depressed level of gonadotropin. A single determination of total 17-ketosteroid excretion provides a poor index of testicular androgen secretion (or total androgenicity), since the normal range is wide and about two-thirds of the total is derived from adrenal androgens, which on the average are considerably less potent than testicular androgens (52; 53, pp. 409 and 434; 54). Administration of estrogen results in a greater decrease in the excretion of androsterone and etiocholanolone than of total 17-ketosteroids, and total 17-ketosteroid excretion during treatment may remain as high as 16 or 18 mg. per twenty-four hours, although regularly decreased from the value before treatment (55). Hence in the present case the preoperative "normal" value of 17.5 mg. per twenty-four hours is compatible with decreased secretion of testosterone by the extra-tumoral testicular tissue; this possibility is supported both by the sparseness of interstitial cells noted on microscopic examination of the testis outside the tumor and by the postoperative rise and maintenance of 17-ketosteroid excretion above the preoperative level.

With regard to the secondary testicular effects of estrogen, certain studies in animals have suggested that the germinal epithelium is more sensitive than the interstitial cells (40, 41), but in studies of human subjects it has usually been found that both varieties of cells are affected (42). In the present case, both types of effect were definite but incomplete. Despite enough tubular degeneration to cause testicular atrophy, there was retention of fertility and decreased but active spermatogenesis; despite a decrease in the number of interstitial cells and in some androgenic effects, libido and potency were normal and unchanged. These facts imply that the

[3] The tubules comprise over 90 per cent of the testicular mass.

amount of estrogen secreted by the tumor was moderate, as indicated by the results of bioassay.

The negative results of "qualitative" (high-threshold) tests for chorionic gonadotropin are in agreement with the results in all tested prepubertal (5–8, 10–12, 17) or adult patients, either with (4, 26, 28, 30, 33, 35–37, 39)[1] or without (56, 57) gynecomastia, with the exception of 1 doubtful[1] case (3). The normal excretion of 17-hydroxycorticoids was also in accord with the reported data on 2 prepubertal (15, 22) and 3 adult (4, 36, 38) cases in which excretion of adrenal corticosteroids was determined.

Endocrine changes following excision of the tumor. Neither gonadotropin nor estrogen determinations were obtained soon after operation, and the assays at seven weeks postoperatively did not use dilutions high enough to include a negative end-point. Nevertheless, the rapid subsidence of gynecomastia, the prompt appearance of an increase in clinical androgenic manifestations, and the marked rise in 17-ketosteroid excretion indicate that the estrogen level must have fallen precipitously and that pituitary gonadotropin secretion must have increased promptly and vigorously. Operative stress would not account for this rise in 17-ketosteroid excretion (58). For many months after orchiectomy, gonadotropin excretion remained at the upper limit of normal and 17-ketosteroid excretion continued well above preoperative levels. The progressive enlargement of the contralateral testis to normal size during this period also reflects gonadotropic activity.

The plateau, or the distinctly slow decline, of estrogen excretion for at least four months after operation raises the possibility of pituitary gonadotropic stimulation of testicular secretion of estrogen; no proof of this possibility has been reported yet, although evidence has been presented to show that chorionic gonadotropin stimulates estrogen secretion by the testis, independently of androgen secretion (52, 54). The increased secretion of androgen postoperatively in this case may also be implicated, since some transformation of androgen to estrogen is known to occur (52; 53, p. 89; 59). Increased secretion of estrogen by the adrenal cortex, due to ACTH stimulation aroused by operative stress, might also have been a factor. It has been shown that administration of exogenous ACTH stimulates secretion of estrogen by the adrenal (60, 61), and excretion of estrogen in some postmenopausal women has been found to rise after bilateral ovariectomy and then decline very slowly (62, 63). However, the significance of an adrenal contribution of estrogen in the present case is uncertain, since no elevation of 17-hydroxycorticoid excretion was revealed by simultaneous determinations, and the operative stress was minor except for general anesthesia.

The recurrence of gynecomastia several months after operation could be

related to the prolonged slightly elevated excretion of estrogen, whatever its cause, or to the direct action of increased androgen; McCullagh and Rossmiller (64) observed gynecomastia during the treatment of eunuchoid patients with methyltestosterone, which is not metabolized to estrogen (52, 54). The gynecomastia frequently seen during puberty similarly poses a question of mechanism (53, p.259; 65; 66). The ultimate subsidence of recurrent gynecomastia, the decline of gonadotropin excretion, and the return of estrogen excretion to normal confirm the supposition that the postoperative endocrine changes were physiologic. No clinical or x-ray evidences of recurrence or metastasis were found, in agreement with past experience: although a few interstitial-cell tumors in adults have proved malignant, all reported interstitial-cell tumors causing clinical endocrine disturbances have been benign, except for 1 doubtful case (3).

The postoperative changes in the present patient are spoken of figuratively as "second puberty," in order to emphasize their resemblance, clinically and chemically, to the events of normal puberty (48, p.265; 53, p.259; 65) and the common primary mechanism of increased pituitary gonadotropin output. The differences in this case can be explained by 1) the original presence of excessive estrogen, in contrast to the rise from very low levels as puberty develops, 2) the sudden increase of gonadotropin secretion due to excision of the estrogen-producing, and hence gonadotropin-suppressing, tumor, and 3) the fact that most of the virilization had occurred before the "pubertal" process postoperatively in this case.

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Letters to the Editor

THE EFFECT OF *dl*-3,3′,5′-TRIIODOTHYRONINE IN GRAVES' DISEASE

To the Editor:

Pittman and Barker (1) have reported suppression of the increased oxygen consumption produced by *l*-thyroxine in thyroidectomized rats during the administration of *dl*-3,3′,5′-triiodothyronine[1] (reverse T_3). The dose of reverse T_3 was 200 times that of thyroxine (by weight) in that study. Pittman and coworkers (2) have administered dosages as large as 20 mg. of reverse T_3 per day during a 19-day study of a hyperthyroid patient. The BMR was +34 per cent before therapy and was +29 per cent at the conclusion of treatment. Because some hyperthyroid patients secrete as much as 900 μg. of hormonal iodine per day (3), or the equivalent of 1.4 mg. of thyroxine, it seemed possible that dosages of reverse T_3 in the range of 200 mg. per day might be required to decrease the BMR in some patients with Graves' disease.

A 53-year-old man with typical Graves' disease was admitted to Memorial Center on March 9, 1959. The level of serum protein-bound iodine (PBI) was 10.4 μg.; total iodine (TI), 10.5 μg.; and total cholesterol (4), 81 mg. per 100 ml. The thyroidal uptake of radioiodine was 80.1 per cent at twenty-four hours, and the levels of plasma I^{131} and PBI^{131} were respectively .44 and .32 per cent per liter of serum at twenty-four hours.

After three control days, the patient was given 10 mg. of reverse T_3 orally every six hours for one day. The dosage was then increased to 40 mg. every eight hours. On the fourth day of administration the radioiodine uptake in the thyroid was 28.9 per cent at twenty-four hours. On the thirteenth day of treatment, 40 mg. of reverse T_3 were administered and serial determinations were made of serum PBI and TI concentrations for the next twenty hours. A maximum elevation of the PBI level to 11.8 μg. and of the TI level to 14.4 μg. per 100 ml. was observed at one hour. At the end of twenty hours without further administration of reverse T_3, the concentration of PBI decreased to 10.3 μg. and that of TI to 12.0 μg. per 100 ml. These changes were barely more than the limits of variation of the method (5).

[1] Kindly supplied by Drs. Michael C. Barry and Robert I. Meltzer, Warner-Lambert Research Institute, Morris Plains, New Jersey.

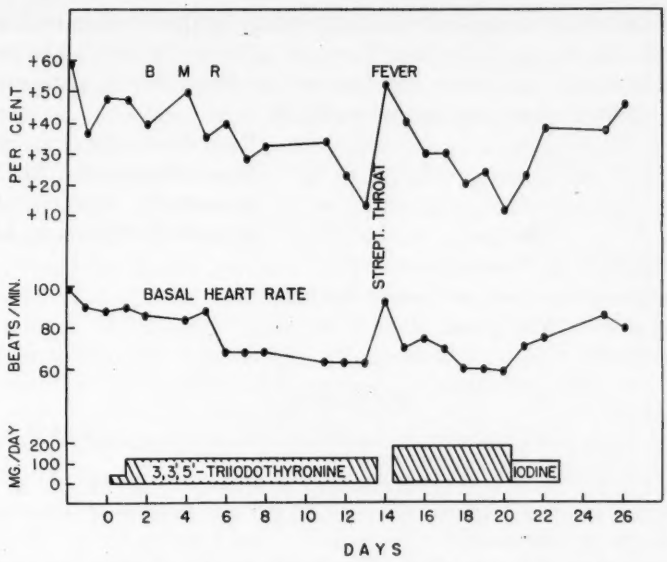

FIG. 1. Changes in basal metabolic rate and heart rate during administration of 3,3′,5′-triiodothyronine in a case of Graves' disease.

The changes in the basal heart rate and metabolic rate, determined by one of us (S.K.) at the bedside, are shown in Figure 1. Serum cholesterol concentration rose slightly to 120 mg. per 100 ml. on the thirteenth day. There was no significant change in the body weight or in the degree of toxicity, as judged clinically, throughout the study. On the fourteenth day after the administration of reverse T_3 was started, while the drug was omitted to obtain the PBI curve, streptococcal pharyngitis with fever developed. The fever responded to penicillin therapy in one day, but penicillin was continued for the duration of the study. At the same time the dosage of reverse T_3 was increased to 30 mg. every four hours until the twentieth day. Immediately thereafter the patient was given 100 mg. of iodide (as KI) daily in fractional doses. Iodide was discontinued two days later to permit radioiodine therapy.

Reverse T_3, in the large dosages used, produced an apparently significant decrease in the BMR (from +48 to +13 per cent) and in the pulse rate. This drug has been reported to be rapidly removed from the circulation and to be at least partially deiodinated (6). The very slight rise in the levels of PBI and TI in the serum following the ingestion of 40 mg. of reverse T_3 confirms these observations. The tendency of the BMR and pulse rate to increase when equivalent amounts of iodide were given at the con-

clusion of the study is evidence against explaining the changes on the basis of an iodide effect alone. Reverse T_3 seems to be well tolerated in the dosages administered and warrants further investigation in patients with hyperthyroidism when it is again available.

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SUBCORTICAL STIMULATION OF THE BRAIN AND RELEASE OF ANTIDIURETIC HORMONE IN MAN

TO THE EDITOR:

Andersson (1) has demonstrated neurohypophyseal secretion by means of electrical stimulation of the anterior hypothalamus of goats, but the afferent neural pathways from higher centers of the brain to the hypothalamus regulating the release of antidiuretic hormone (vasopressin, ADH) from the neurohypophysis are virtually unknown.

Heath and co-workers of the Department of Psychiatry and Neurology, Tulane Medical School, have investigated the therapeutic effects of electri-

cal stimulation of various areas of the brain of patients with schizophrenia and temporal-lobe epilepsy (2). Approximately two years ago, we had the unique opportunity[1] to evaluate the antidiuretic responses to stimulation of various subcortical areas of the brain of the last patient studied by this group in recent years. These observations demonstrated for the first time neural pathways regulating release of ADH in man. Since more extensive studies of this nature may not be forthcoming in the immediate future, publication of these preliminary observations appears warranted.

The patient, a 28-year-old man, drank 1.0 liter of tap water initially, and additional tap water at intervals thereafter to maintain a positive water balance of 1.0 liter throughout each study. Urine was collected through an indwelling catheter while the patient was recumbent. The electrical stimulus was applied as a biphasic rectangular pulse wave of 1.0 millisecond's duration, with 50 pulse pairs per second. The following subcortical areas were stimulated: *a*) septal region (2), unipolar electrode, maximum of 10 milliamperes and 12 volts, continuous for 14.5 minutes; *b*) same septal electrode, maximum of 10 milliamperes and 12 volts, continuous for 15 minutes; *c*) left amygdaloid and posterior hippocampus, bipolar electrodes, maximum of 7 milliamperes and 18 volts, 6 minutes' duration, discontinued for 2.5 minutes and then reinstituted for an additional 9.33 minutes to a maximum of 10 milliamperes and 20 volts; *d*) right amygdaloid and posterior hippocampus, bipolar electrodes, maximum of 8 milliamperes and 15 volts continuous for 15 minutes.

Urine and plasma were analyzed for total solute concentration (Fiske osmometer), sodium and potassium (Baird flame photometer), and creatinine (3). Acute changes in urinary total solute concentration (U_{osm}) and free water clearance (C_{H_2O}) were utilized as indices of ADH release (4).

Faradic stimulation of the septal region produced similar antidiuretic responses on two occasions (Table 1). Stimulation of the left amygdaloid nucleus, which was begun on the ascending limb of the initial water diuresis, resulted in only a slight antidiuretic response (Table 1) manifested by transient blunting of the peak of the diuresis. Stimulation of the right amygdaloid nucleus produced a greater rise in U_{osm} and a more prolonged antidiuretic response than had been observed with septal stimulation (Table 1, Fig. 1).

These antidiuretic responses were comparable to those produced by stimulation of the neurohypophysis of normal subjects with nicotine (4), and approximated the effect of physiologic doses of intravenous Pitressin.

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[1] We wish to thank Dr. R. G. Heath for allowing us to make these physiologic observations during the course of his investigations.

TABLE 1. ANTIDIURETIC RESPONSES

	C_{H_2O} (ml./min.)	$U_{osm.}$ (mOsm./Kg.)	Duration of antidiuresis (mins.)
Septal region			
before stim.	+16.1	46	
after stim.	− 0.4	318	90
Pitressin, 5 milliunits i.v.	+ 6.9	90	15
Septal region			
before stim.	+18.8	45	
after stim.	+ 0.2	254	80
Lt. amygdaloid			
before stim.	+10.2	79	
after stim.	+ 9.0	87	30
Rt. amygdaloid			
before stim.	+16.9	58	
after stim.	− 2.5	557	110

Stimulation of these areas of the brain had no effect on clearance of creatinine or excretion of sodium, potassium and total solute.

The patient was under significant emotional stress and complained of ill-

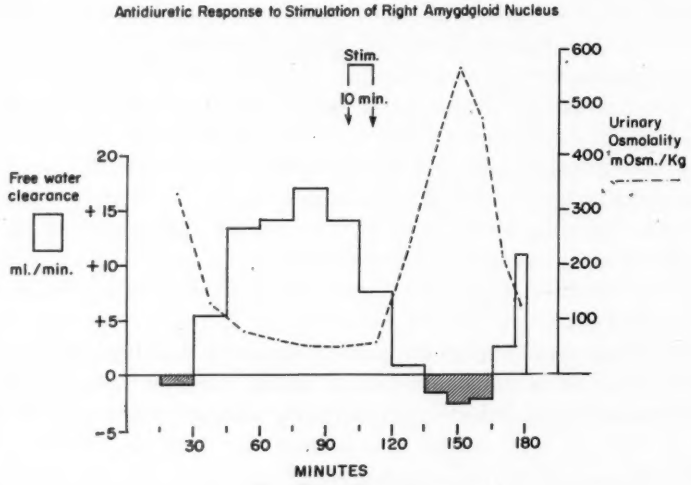

FIGURE 1

defined apprehension and buzzing sounds in the head during application of the electrical stimulus. Transient increases in pulse rate and blood pressure were the only objective changes during stimulation.

Appropriate stimulation of the left amygdaloid nucleus probably would have also invoked a significant antidiuretic response; the electrodes may not have been placed in close enough contact with neuronal masses or fiber tracts communicating with the supraoptic nuclei, or the temporary interruption of stimulation and shorter period of continuous application may have prevented proper activation of the neurohypophysis. Since the autonomic and psychic responses in this study were as intense as those observed in the remainder, it is unlikely that the antidiuretic responses were mediated through nonspecific effects of emotional stress or autonomic stimulation on neurohypophyseal secretion.

These observations[2] suggest that certain subcortical centers of the brain may play an important selective role in the neural control of the release of ADH in man. These tentative conclusions, based upon studies of a single human subject, have been investigated recently in similarly prepared rhesus monkeys (5), and the results to date tend to corroborate the observations reported here.

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[2] Supported, in part, by grants from the USPHS (H2850), and the Louisiana and American Heart Associations.

FALSELY ELEVATED VALUES FOR URINARY 17-KETOSTEROIDS AND 17-HYDROXYCORTICOIDS ASSOCIATED WITH INGESTION OF TRIACETYLOLEANDOMYCIN

To the Editor:

An adolescent girl with acne, but with no other feature suggestive of adrenocortical hyperfunction, was noted to have elevated urinary excretion of 17-ketosteroids and 17-hydroxycorticoids. Furthermore, upon analysis of the urine, the final color of the hydroxycorticoid reaction was brownish rather than yellowish. Since the only medication being taken by the patient was triacetyloleandomycin with glucosamine (TAO), the following studies

Table 1

Subject	TAO (Gm./day)	Values for urinary excretion	
		17-KS* (mg./day)	17-OH-CS† (mg./day)
a. A.G.	1	16.8	15.9
	1	11.7	16.1
	0 (1 mo.)	13.9	9.3
	0 (2 mos.)	6.7	3.5
b. M.P.	0	17.7	6.6
	1	19.0	15.5
	2	23.4	27.3
M.N.	0	13.4	6.6
	1	21.5	15.5
	2	23.7	27.3
	0 (1 mo.)	12.0	5.5
	¼	11.4	5.6
S.S.	0	17.5	4.4
	2	18.9	23.5
c. Addison's disease	0	4.4	3.6
	2	18.9	23.5
d. Control	0	9.7	2.9
	¼ Gm. added to urine	18.0	10.5

* Determined by the method of Drekter *et al.*—normal range 4–14 mg. for women and 8–20 mg. for men.

† Determined by the method of Silber and Porter—normal range 3–10 mg. for both sexes.

were performed to ascertain whether the administration of this antibiotic could account for these observations.

The urinary excretion of steroids by the subject mentioned (A.G.), during and after the ingestion of TAO, is presented in Table 1(*a*). The elevated excretion fell to normal after cessation of ingestion of the antibiotic.

Three normal adult males collected urine specimen before and after the ingestion of TAO for two days at each of several dosage levels. The data in Table 1(*b*) demonstrate a marked rise in the urinary 17-hydroxycorticoids and a moderate rise in the 17-ketosteroids during the daily administration of 1 or more grams of this antibiotic.

To exclude a stimulatory effect of TAO upon the adrenal cortex, the study was repeated in a patient who had had a bilateral adrenalectomy and who was taking a maintenance dosage of 20 mg. of hydrocortisone per day. It will be noted in Table 1(*c*) that the administration of TAO resulted in an increased excretion of both 17-ketosteroids and the 17-hydroxycorticoids to many times the control level. Adrenal stimulation can thus be excluded as a mechanism for the observed results.

An *in vitro* study was performed in which TAO was added directly to a 24-hour collection of urine in order to determine whether the antibiotic itself could account for the elevation of urinary steroid values. In Table 1(*d*) the data are listed which indicate that this antibiotic was the direct cause of the elevation.

The administration of TAO to human subjects resulted in an elevation of urinary 17-ketosteroid and 17-hydroxycorticoid excretion. The effect was not dependent upon the presence of the adrenal glands. This antibiotic acts as a chromogen with the reagents used in measuring the values of steroid excretion and thus gives the false impression of increased adrenocortical activity.

Acknowledgment

The authors are indebted to Dr. Lawrence Frank for the opportunity of studying the first patient, and to Dr. Carlozzi of Pfizer Laboratories for providing the TAO.

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HYDROLYSIS OF ESTROGEN CONJUGATES IN DIABETIC PREGNANCY URINES*

To the Editor:

The most acceptable conditions for the acid hydrolysis of urinary estrogen conjugates, namely, boiling the urine with 15 volumes per cent of concentrated hydrochloric acid for sixty minutes under reflux (1), have been incorporated into 2 reliable procedures for measuring urinary estriol, estrone and estradiol-17β (2, 3). Even under these circumstances, destruction of estrogen still occurs, but it may be minimized by diluting the urine with water prior to hydrolysis (4).

One substance reported to increase destruction of estrogen during acid hydrolysis is glucose (5). In this laboratory, preliminary findings in a study of estrogens in diabetic pregnancy indicated that estriol, estrone and estradiol excretion was markedly low in a number of cases. Because of the difficulties involved in acid hydrolysis and the possible effect of urinary glucose on recovery of estrogen from urine, we carried out the investigation reported here.

MATERIALS AND METHODS

Complete 24-hour urine specimens were collected and stored at 5° C. or in a deep-freeze until analyzed. Under these conditions there was no loss of estriol, estrone or estradiol during the storage period. Determinations of sugar were semiquantitative, using Clinitest tablets (Ames Co. of Canada, Ltd., Toronto).

Acid hydrolysis was carried out on 100-ml. samples of undiluted urine, by the method of Bauld (3). For diluted urine (mid or late pregnancy), samples containing 50, 25 or 10 ml. were diluted to 100 ml. with water to yield dilutions of 1:2, 1:4 and 1:10 respectively, prior to acid hydrolysis. For a 1:20 dilution, a 25-ml. aliquot of urine was diluted to 500 ml. For early pregnancy or other urines with low estrogen levels, a 50-ml. aliquot was diluted to 500 ml. to yield a 1:10 dilution.

For enzyme hydrolysis, a 10-ml. or 20-ml. volume of mid or late pregnancy urine was adjusted to pH 6.5 (glass electrode) with dilute sodium hydroxide or acetic acid.

* Supported by grants from Ayerst, McKenna & Harrison Limited; The Banting Research Foundation; Parke, Davis and Company; and the Women's Auxiliary of The Montreal General Hospital.

TABLE 1. ESTROGEN LEVELS AS MEASURED IN DIABETIC PREGNANCY URINES USING ACID HYDROLYSIS AFTER DIFFERENT DEGREES OF DILUTION OF THE URINE

(Results expressed as μg. per 24 hours)

Urine specimen	Estrogens	Degree of dilution of urine			
		1:2	1:4	1:10	1:20
M_1*	estriol	4,840		9,610	
	estrone	89		119	
	estradiol	47		122	
M_2*†	estriol	5,466		9,923	
	estrone	194		306	
	estradiol	84		196	
M_3*	estriol		9,600	8,020	
	estrone		114	116	
	estradiol		106	87	
L_1	estriol		4,160	5,760	6,360
(½% sugar)	estrone		95	132	92
	estradiol		60	90	104
B_1	estriol		9,920	12,300	
(½% sugar)	estrone		774	858	
	estradiol		124	136	

* Sugar content not measured.

† Premarin (estrogen conjugate preparation—Ayerst, McKenna & Harrison, Montreal) in a dosage of 15 mg. daily was administered to the patient at this time.

This was incubated at 37° C. for twenty-four hours with 20 units of bacterial β-glucuronidase[1] per ml. of original urine. After cooling, the solution was diluted to 100 ml. for extraction. The conditions of incubation were chosen on the basis of results obtained in comparative experiments (unpublished), in which acid and enzyme hydrolysis were employed.

The chemical determination of estriol, estrone and estradiol was made in duplicate by a modification of Bauld's method (3). This involved omitting saponification of the estrone and estradiol fractions after chromatography, and dispensed with chromatography of the estriol fraction after saponification. This is permissible when estrogen levels are high. Average recoveries of estriol, estrone and estradiol added to normal urine after acid hydrolysis are 80, 90 and 90 per cent respectively, by this procedure.

RESULTS

Table 1 shows striking differences in estrogen levels between 1:2 and

[1] Glucuronidase of bacterial origin (Sigma Chemical Co., St. Louis, Missouri). The activity is expressed in Sigma units as defined by the manufacturers.

TABLE 2. COMPARISON OF ACID AND ENZYME HYDROLYSIS OF ESTROGEN CONJUGATES IN DIABETIC PREGNANCY URINES

(Results expressed as μg. per 24 hours)

Urine specimen	Estrogen	Acid hydrolysis		Enzyme hydrolysis
		No dilution	1:10 dilution	
M_4* (2% sugar)	estriol	2,310	11,440	11,000
	estrone	264	986	990
	estradiol	33	360	646
M_5† (2% sugar)	estriol	3,480	16,278	14,920
	estrone	195	267	279
	estradiol	40	157	159
L_2 (2% sugar)	estriol	5,750	15,900	16,750
	estrone	113	142	163
	estradiol	100	144	125
L_3 (no sugar)	estriol	14,000	16,459	17,000
	estrone	175	166	163
	estradiol	150	148	125
		(1:2.5)‡	(1:25)	
S (no sugar)	estriol	15,350	22,115	17,750
	estrone	300	470	318
	estradiol	216	363	320

* Patient received 15 mg. of Premarin per day (see Table 1).

† Patient received 175 mg. of stilbestrol per day.

‡ Urine S was diluted to 2500 ml. before acid hydrolysis; this resulted in a dilution of 1:2.5.

1:10 dilution (urines M_1 and M_2) but no such variation between 1:4 and 1:10 for another urine, M_3. In 2 other urines (L_1 and B_1) there were apparent differences between 1:4 and 1:10 dilutions. Also, an increase in estriol was observed when urine L_1 was diluted from 1:10 to 1:20, although estrone and estradiol values become inaccurate at the higher dilution. The very large differences which may occur in estrogen levels between undiluted urines, and urines diluted 1:10 before acid hydrolysis, are particularly obvious in Table 2 (urines M_4, M_5 and L_2). The much smaller effect of dilution for urine L_3 (no sugar) suggests the relationship of urinary sugar to destruction of estrogen. Although in urine S, with no sugar, there was an increase in all 3 estrogens upon dilution, it should be noted that the lower dilution in this case was 1:2.5 and the higher, 1:25.0. In general, estrogen

TABLE 3. EFFECT OF ADDED GLUCOSE ON THE ACID HYDROLYSIS OF URINARY ESTROGEN CONJUGATES IN NORMAL AND DIABETIC PREGNANCY URINES

(Results expressed in μg. per 24 hours)

Urine specimen*	Degree of dilution	Estriol	Estrone	Estradiol
S	1:2.5	15,350	300	300
S	1:25	22,115	470	363
S+5% glucose	1.2.5	6,610	140	87
S+5% glucose	1:25	17,600	323	198
H	1:10	8,963	1,792	387
H+5% glucose	Nil	1,353	401	44
H+5% glucose	1:10	7,255	1,444	333

* S = diabetic pregnancy urine containing no sugar.
H = normal pregnancy urine.

excretion values after enzyme hydrolysis showed good correlation with those after acid hydrolysis of the 1:10 diluted urines M_4, M_5, L_2 and L_3 (Table 2). For urine S (1:25 dilution) lower values were found after enzyme hydrolysis than after acid hydrolysis.

Table 3 shows that the addition of 5 per cent glucose to pregnancy urine prior to acid hydrolysis resulted in loss of estrogen. Also, this could be largely, if not wholly, compensated for by a tenfold dilution with water prior to hydrolysis.

DISCUSSION

It is apparent that dilution of diabetic pregnancy urine before acid hydrolysis results in a general increase in values for estriol, estrone and estradiol excretion. The extent of this increase seems to be variable for the 3 estrogens. Thus in 3 urines with similar sugar content (M_4, M_5 and L_2; Table 2), acid hydrolysis after 1:10 dilution resulted in an increase varying from 30 per cent (estrone in L_2) to 1100 per cent (estradiol in M_4) over that for undiluted urine. In a sugar-free urine (L_3) there was only a modest increase of estriol (15 per cent) on dilution. This is the expected observation when normal (nondiabetic) urine is diluted 1:10 before acid hydrolysis (4). The greatest destruction of estrogen occurs when acid hydrolysis is performed in undiluted urines containing glucose, and it is evident that urinary estrogen values based on these methods are open to considerable criticism.

The good agreement between estrogen values measured after acid hydrolysis in a 1:10 dilution of urine and those after enzyme hydrolysis, although not constituting proof for completeness of estrogen release, suggests that either procedure is capable of yielding more meaningful results than

those obtained following hot-acid treatment of the undiluted urine. One interesting feature (Table 2) is the significantly lower estriol and estrone levels observed after enzyme hydrolysis than after acid hydrolysis of a 1:25 dilution of urine S. One possible explanation is the presence of estrogen conjugates, other than glucuronides, not hydrolyzed by β-glucuronidase and possibly destroyed during acid hydrolysis at lower dilutions of urine.

It is concluded that any investigation of urinary estrogens (estriol, estrone and estradiol) in diabetic pregnancy requires that hydrolysis of the conjugates be effected either by acid hydrolysis following 1:10 dilution of the urine or by incubation with β-glucuronidase. There can be little doubt that glucose is a major factor in loss of estrogen during acid hydrolysis of undiluted diabetic urines. This suggestion receives considerable support from the data in Table 3.

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Acknowledgments

The authors wish to acknowledge the continued interest of Drs. E. H. Bensley and J. S. L. Browne in this work. Clinical collaboration was provided by Drs. P. R. Blahey and G. E. Joron. We are grateful to Mr. Dan Broida, Sigma Chemical Co., St. Louis, Missouri, for a gift of bacterial β-glucuronidase.

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STIMULATION OF RESPIRATION OF SLICES OF HUMAN ADENOHYPOPHYSIS BY ESTRADIOL-17β *IN VITRO**

To the Editor:

Estradiol-17β added *in vitro* causes an increase in oxygen consumption in human endometrium (1), placenta (2), and mammary gland (3), but not in liver or kidney (4). It has been shown in human placenta that this increased respiration is due to an estradiol-sensitive transhydrogenase in the particle-free supernatant fraction of the cell (5). Estrogens likewise cause an increase of oxygen consumption *in vitro* in the adenohypophysis of the castrated female rat, but not in the kidney or liver (6). It was considered of interest, therefore, to study the effect of estradiol added *in vitro* on the oxygen consumption of slices of human adenohypophysis. This tissue, obtained at operation from patients undergoing hypophysectomy for the palliation of advanced carcinoma of the breast, has been sporadically available to us for the past two years.

METHODS

The adenohypophyses were taken from the operating room immediately after extirpation and chilled in ice-cold 0.9 per cent NaCl. A small mid-sagittal section including the residual stalk and neurohypophysis was removed for study by the pathologist. The remainder was trimmed free of connective tissue, and sliced with a Stadie-Riggs microtome. Tissue slices of 50 to 100 mg. fresh weight were weighed to the nearest tenth of a milligram on a torsion balance and incubated for three hours at 37° C. in small Warburg vessels of approximately 7.5 ml. total volume containing 1.5 ml. of incubation media and air as the gas phase. The incubation medium was a saline-phosphate solution pH 6.9 (1); it contained 0.67 micromole of citrate per vessel. Because of the small amount of tissue available for each experiment, a single control vessel was incubated along with a single vessel containing 4×10^{-6}M of estradiol-17β prepared as previously described (1).

RESULTS AND COMMENT

Estradiol-17β *in vitro*, at a concentration approaching that found *in vivo*, had a striking stimulatory effect on the oxygen consumption of slices of adenohypophysis from women with advanced carcinoma of the breast (Table 1). The difference between the mean values for the contents of the control Warburg vessels and those containing estradiol indicated an average increase of approximately 57 per cent.

Considerable variation was evident in both the base-line respiration and the magnitude of stimulation; however, despite the heterogeneous endocrine histories of the patients prior to hypophysectomy, a stimulation of oxygen consumption by hypophyseal slices could be elicited in 11 of the 12

* These investigations were aided in part by grants from the Charles A. King and Marjorie King Fund and from Grant C2400, National Institutes of Health.

TABLE 1. THE EFFECT OF ESTRADIOL-17β ON THE OXYGEN CONSUMPTION OF SLICES OF HUMAN ANTERIOR PITUITARY GLAND

Peter Bent Brigham Hospital Case No.	Control	Estradiol-17β	Ratio: Estradiol/ Control
3K-964	26*	33	1.26
5L-494	22	37	1.64
8F-427	209	301	1.44
5L-909	112	128	1.15
3M-7	35	86	2.45
3H-674	186	214	1.15
5L-407	32	154	4.79
5M-395	110	60	0.55
3X-261	120	161	1.34
0L-714	137	219	1.59
9M-865	24	148	6.13
3N-463	97	198	2.04
Mean†	92	145	

* All values expressed as microliters of O_2 consumed per gram of wet weight per hour.
† Difference between the means significant at $P < .1$ by paired *t*-test.

cases tested. Two of the women were pregnant at the time of hypophysectomy; one (#0L-714) was in the ninth month of gestation and the other (#3K-964) was in the fifth month. The former case has been reported in detail (7). Eight of the 10 nonpregnant women had been surgically oöphorectomized at varying periods of time prior to hypophysectomy; the other 2 had radiation-induced menopause. One of these 2 women (#8F-427) had a vaginal smear which showed no evidence of estrogenic stimulation, but respiration of pituitary slices was strikingly increased by estradiol *in vitro*. Vaginal smears from the other woman (#5M-395) showed evidence of estrogenic stimulation. This was the only 1 of the 12 cases in which there was no *in vitro* stimulation of respiration; the case was also exceptional in that the time from original diagnosis to hypophysectomy was thirteen years—over twice as long an interval as the next longest one. After referral to the Peter Bent Brigham Hospital, all of the women received cortisone (75 mg. per day) for a variable period of time prior to operation, and hydrocortisone (100 mg.) during the hypophysectomy.

There was no correlation between the magnitude of estrogenic stimulation of respiration of pituitary slices observed *in vitro* and the following clinical aspects of the cases: the age of the patient, the histologic type of the primary lesion, the distribution of metastases, the time of survival after hypophysectomy, and the clinical response to hypophysectomy as inde-

pendently evaluated by Dr. Andrew Jessiman who was in charge of the clinical study. Histologic examination of the pituitaries from these cases revealed no striking pathologic abnormalities.

SUMMARY

Slices of adenohypophysis from patients undergoing hypophysectomy for advanced carcinoma of the breast exhibit stimulation of respiration when estradiol-17β is added *in vitro*. The effect is similar to that demonstrated previously with human endometrium, placenta, and mammary gland.

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Acknowledgments

We are particularly grateful to Drs. Andrew Jessiman, Donald Matson and Francis D. Moore of the Department of Surgery of the Peter Bent Brigham Hospital for making the pituitaries available to us. Dr. Matson performed the hypophysectomies. We are also obliged to Dr. Gustav Dammin and the staff of the Department of Pathology of the Peter Bent Brigham Hospital for their cooperation in obtaining the tissue and performing the histologic examinations.

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* Postdoctoral Fellow of the National Cancer Institute, United States Public Health Service.

The Endocrine Society

THE 1960 ANNUAL MEETING

The Forty-Second Annual Meeting of The Endocrine Society will be held in the Eden Roc Hotel, Miami Beach, Florida, Thursday, Friday and Saturday, *June 9, 10 and 11, 1960.*

The Chairman of Local Arrangements is Dr. Kenneth Savard.

Scientific Sessions will be held from 9:00 A.M. to 5:00 P.M. daily and in addition there will be simultaneous afternoon sessions. The annual dinner is scheduled for Friday, June 10, at 7:30 P.M. preceded by cocktails at 6:30 P.M.

All members are urged to make their hotel reservations immediately. The Eden Roc will hold 250 bedrooms and the adjoining hotels, the Montmartre Hotel and Lucerne Hotel, will hold 75 rooms each for members until May 1, 1960, after which time the hotel will not guarantee further reservations. Therefore it is imperative that you make your reservations early, directly with the hotel, advising them of time and date of arrival and departure. If you plan on remaining through June 12 for the American Medical Association and other meetings, it is imperative that your reservation be made from June 12 on, directly through the A.M.A. Housing Bureau, c/o Miami Beach Convention Bureau, 1700 Washington Avenue, Miami Beach, Florida, since no reservation beyond June 12 can be accepted directly by the hotels. Make your reservations early and avoid disappointment. Hotel reservation card will be mailed to members shortly after January 1, 1960.

Final program, membership card and advance registration forms will be sent on May 1, 1960 to members whose current dues have been paid.

Those wishing to present papers, which will be strictly limited to ten minutes, should send an original and 4 copies of the title and abstract to the Vice-President, Dr. Robert H. Williams, University of Washington School of Medicine, Seattle 5, Washington, *not later than February 15, 1960.* It is imperative that the abstracts be informative and complete with results and conclusions—not a statement that these will be presented at the meeting—in order that they may be of reference value and suitable for printing in the program. The reading and processing of approximately 200 abstracts submitted each year and compiling of the program from these involves a tremendous amount of time and effort on the part of the

Program Committee. THE COUNCIL REQUESTS THAT AUTHORS ADHERE STRICTLY TO THE FOLLOWING RULES WHEN SUBMITTING ABSTRACTS; OTHERWISE THEY CANNOT BE CONSIDERED:

1. IT IS ASSUMED THAT ABSTRACTS SUBMITTED FOR THIS PROGRAM HAVE NOT AND WILL NOT BE SUBMITTED ELSEWHERE.
2. Abstracts may not exceed 200 words, or equivalent space, exclusive of title. No footnotes or acknowledgments to sponsors can be published. References, if used, must be placed in the body of the text. The abstract should consist of a single paragraph, if possible. Structural chemical formula cannot be used.
3. The title heading must be arranged as follows:
   Line 1. Title, not to exceed 15 words;
   Line 2. Author/s. The name of each nonmember-author collaborating with member-authors is to be followed by the phrase "(by invitation)." Names of nonmembers who are introduced, *i.e.*, who are not collaborators with member-authors, are to be followed by the phrase "(introduced by . . .)." The principal degree, *e.g.*, M.D., of each author should be written after his name.
   Line 3. Institution of origin and city in which institution is located.
4. Authors who wish receipt of their abstracts acknowledged should enclose self-addressed post card with the title of the abstract noted thereon.
5. Authors are also to enclose 3×5 inch filing cards, one for each author, giving his name and initials in the upper right-hand corner, and the complete title of the abstract with all the authors listed in the center of the card. There should be a second card enclosed listing the title, all authors, and the mailing address of the senior author. This is necessary for indexing. Senior authors will be notified of the acceptance of their papers for the final program.
6. The body of the abstract, typed double space, should follow the heading. The original copy should be on bond paper. The original and 4 copies should be forwarded to Dr. Williams.
7. Abstracts should be letter perfect, since there will be no opportunity for proof reading by the authors.

THE 1960 AWARDS

The selection of the recipients of the awards of The Endocrine Society is made by a Committee appointed by Council of the Society. These awards and fellowships carry no obligation by the recipient to the Society or to the donors.

FRED CONRAD KOCH AWARD

In 1957 a substantial legacy was bequeathed to the Society by the late Elizabeth Koch for the purpose of establishing the Fred Conrad Koch Memorial Fund in memory of her late husband, Distinguished Service Professor of Physiological Chemistry at the University of Chicago and pioneer in the isolation of the androgens. This is the highest honor of The Endocrine Society and is represented by a medal known as the Koch Medal of The Endocrine Society. The medal, as well as an honorarium of $3,500, is to be given annually to an individual for work of special distinction in endocrinology. The recipient shall be chosen from nominations presented by members of the Society and is limited to citizens of the United States and Canada.

This Award was first presented in 1959, to Dr. W. W. Swingle. The Koch Medal has replaced the Medal of The Endocrine Society which was established in 1954 and presented to Dr. Carl R. Moore in 1955, Dr. Frederick L. Hisaw in 1956, Dr. Joseph C. Aub in 1957 and Dr. I. L. Chaikoff in 1958. The Medal of the Endocrine Society replaced the E. R. Squibb Award which was formerly the highest honor bestowed by the Society. Past recipients of the Squibb Award were Dr. George W. Corner in 1940, Dr. Philip E. Smith in 1941, Dr. Fred C. Koch in 1942, (no award in 1943), Dr. E. A. Doisy in 1944, Dr. E. C. Kendall in 1945, Dr. Carl G. Hartman in 1946, Drs. Carl F. and Gerty T. Cori in 1947, Dr. Fuller Albright in 1948, Dr. Herbert M. Evans in 1949, Dr. C. N. H. Long in 1950, Dr. J B. Collip in 1951, Dr. James H. Means in 1952 and Dr. David Marine in 1953.

THE CIBA AWARD

The Ciba Award, to recognize the meritorious accomplishments of an investigator not more than 35 years of age in the field of clinical or preclinical endocrinology, was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; 1945—Dr. Jane A. Russell; 1946—Dr. Martin M. Hoffman; 1947—Dr. Choh Hao Li; 1948—Dr. Carl Heller; 1949—Dr. George Sayers; 1950—Dr. Oscar M. Hechter; 1951—Dr. Albert Segaloff; 1952—Dr. Seymour Lieberman; 1953—Dr. Sidney Roberts and Dr. Clara Szego (Mrs. Roberts); 1954—Dr. Isadore M. Rosenberg; 1955—Dr. Jack Gross; 1956—

Dr. Alfred M. Bongiovanni; 1957—Dr. Nicholas S. Halmi; 1958—Dr. Monte A. Greer; 1959—Dr. Gordon L. Farrell. Prior to 1952 the Award was $1,200. It has now been increased to $2,500.

THE AYERST AND THE SQUIBB FELLOWSHIPS

The Ayerst Fellowship was established in 1947 and the Squibb Fellowship in 1956. They are designed to assist men or women of exceptional promise in furthering their advancement towards a career in endocrinology. Each Fellowship is awarded on alternate years and the stipend, which will not exceed $5,000, may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointees. Individuals possessing the M.D. or Ph.D. degree, or candidates for either of these degrees, are eligible for appointment.

Applicants must submit the following information:

1. Evidence of scientific ability as attested by studies completed or in progress.
2. Recommendations from individuals familiar with the candidate and his work.
3. A proposed program of study.
4. Acceptance of the individual by the head of the department in which the Fellowship will be held.
5. A statement that he or she will serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be spent in course work or participation in teaching, the latter purely on a voluntary basis.

THE UPJOHN SCHOLAR OF THE ENDOCRINE SOCIETY

The Council of The Endocrine Society has established a category of Scholars. A grant has been made available through the generosity of the Upjohn Company, and will be awarded to an established investigator or teacher in the field of endocrinology who wishes to extend the opportunities for work either in this country or abroad.

The award will not exceed $2,500 annually and will be granted on the basis of proposals submitted by the applicant. Such applications should include the estimated financial needs. The funds may be used for travel, maintenance and other expenses.

Nominations

Nominations for the Fred Conrad Koch Award, the Ciba Award, and the Ayerst and the Squibb Fellowships may be made by any member of The Endocrine Society. They should be submitted on forms which may be

obtained from the Office of the Secretary, 1200 North Walker Street, Oklahoma City 3, Oklahoma. Completed nominations should be returned to the Secretary not later than *October 15* each year.

Proposals for appointments as a Scholar of The Endocrine Society should be made in writing by the individual, and addressed to the Secretary of the Society. They should be submitted by *October 15* each year.

ELEVENTH POSTGRADUATE ASSEMBLY IN ENDOCRINOLOGY AND METABOLISM

SPONSORED JOINTLY BY THE ENDOCRINE SOCIETY AND THE UNIVERSITY OF CALIFORNIA SCHOOL OF MEDICINE AND DEPARTMENT OF CONTINUING MEDICAL EDUCATION

SAN FRANCISCO, CALIFORNIA

November 9 through 13, 1959

For information concerning the program and registration, write to *Seymour M. Farber, M.D.*, Assistant Dean in charge of Continuing Medical Education, University of California Medical Center, San Francisco 22, California.

The registration fee is $100 payable to the Regents of the University of California. Residents and Fellows will be admitted for a reduced fee of $30. Those wishing to reserve rooms should communicate directly with the Whitcomb Motor Hotel, 8th and Market St., San Francisco.

(For program, see the June issue of the Journal)

The American Goiter Association

THE 1960 ANNUAL MEETING

The next Annual Meeting of the American Goiter Association will be held in *London, England, July 5–9, 1960,* in the Great Hall of the Royal College of Surgeons. This meeting, co-sponsored with the London Thyroid Club, will be the Fourth International Goiter Conference. For further information write Dr. Selwyn Taylor, 3 Roedean Crescent, Roehampton, London, S.W.15, England.

JOHN C. MCCLINTOCK, *Secretary,*
149½ Washington Avenue,
Albany 10, N.Y.

SUBMISSION OF ABSTRACTS FOR THE PROGRAM OF THE FOURTH INTERNATIONAL GOITER CONFERENCE

Abstracts of American papers to be considered for presentation at the Fourth International Goiter Conference, London, England, July 5–9, 1960, should be sent to Dr. J. E. Rall, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda 14, Maryland. Abstracts from all other countries are to be sent to Dr. Selwyn Taylor, 3 Roedean Crescent, Roehampton, London, S.W.15, England.

All American abstracts must be received by Dr. Rall *by December 1, 1959.* They should not exceed 400 words and should be submitted in quintuplicate. Presentation of papers will be limited to fifteen minutes and a copy of the final paper must be sent to the London Secretary two weeks prior to the meeting.

THE VAN METER PRIZE AWARD FOR 1960

The American Goiter Association again offers the Van Meter Prize Award of $300.00 to the essayist submitting the best manuscript of original and unpublished work concerning "Goiter—especially its basic cause." The studies so submitted may relate to any aspect of the thyroid gland in all of its functions in health and disease. The Award will be made at the Fourth International Goiter Conference in London, England, July 5–9, 1960, where a place on the program will be reserved for the winning essay-

ist if he can attend the meeting. For 1960, the recipient of the Award will receive consideration for an award of a travel honorarium.

The competing essays may cover either clinical or research investigations, should not exceed 3,000 words in length and must be presented in English. Duplicate typewritten copies, double spaced, should be sent to the Secretary, Dr. John C. McClintock, 149½ Washington Avenue, Albany 10, New York, *not later than January 1, 1960.* The committee who will review the manuscripts is composed of men well qualified to judge the merits of the competing essays.

SYMPOSIUM ON PARATHYROID RESEARCH TRENDS

A symposium devoted entirely to problems of parathyroid physiology and malfunction will be held at the Rice Institute, Houston, Texas, *February 3–6, 1960.* All interested persons are invited to attend. There are no registration fees; however, pre-registration is requested in order that appropriate facilities can be arranged. The Rice Institute Biology Office will be glad to make hotel or dormitory arrangements for those desiring this service.

PROGRAM

Committee: D. H. Copp, Roy O. Greep, W. F. Neuman, and Roy V. Talmage

WEDNESDAY, FEBRUARY 3, 1960

1:00–6:00 P.M.

Registration and tours of Rice Institute and Texas Medical Center

7:45 P.M.

Welcome by W. V. Houston, President of Rice Institute

8:00 P.M.

Opening Address by Franklin C. McLean, University of Chicago. Introduced by A. B. Hastings, Scripps Clinic and Research Foundation

"Unsolved Problems of Parathyroid Physiology"

THURSDAY, FEBRUARY 4, 1960

9:00 A.M.

PURIFICATION AND CHARACTERIZATION OF PARATHYROID HORMONE

Chairman: *Paul L. Munson, Harvard University School of Dental Medicine*

2. G. Aurbach, National Institutes of Health
Purification of Parathyroid Hormone Following Extraction with Phenol

3. Howard Rasmussen, Rockfeller Institute
   CHEMISTRY OF THE PARATHYROID HORMONE
4. Paul L. Munson, Harvard University School of Dental Medicine
   THE BIOLOGICAL ASSAY OF THE PARATHYROID HORMONE

2:00 P.M.

SELECTED TOPICS IN PARATHYROID PHYSIOLOGY

Chairman: *D. Harold Copp, University of British Columbia*

5. Felix Bronner, Cornell University School of Medicine
   PARATHYROID EFFECTS ON SULPHATE METABOLISM: INTERRELATIONSHIPS WITH CALCIUM
6. M. R. Shetlar, Oklahoma Medical Research Foundation
   EFFECTS OF PARATHYROID EXTRACT ON GLYCOPROTEIN AND MUCOPOLYSACCHARIDE COMPONENTS OF SERUM AND TISSUE
7. Carl Cramer, University of British Columbia
   PARATHYROID INFLUENCE ON CALCIUM ABSORPTION BY THE GUT
8. Leon Kraintz, University of Texas Dental Branch
   RELATIONSHIP OF SALIVARY GLANDS TO PARATHYROID FUNCTION

FRIDAY, FEBRUARY 5, 1960

9:00 A.M.

RELATIONSHIP OF THE PARATHYROIDS TO KIDNEY AND BONE

Chairman: *Roy O. Greep, Harvard University School of Dental Medicine*

9. F. C. Bartter, National Institutes of Health
   PARATHYROID INFLUENCES ON PHOSPHATE EXCRETION
10. Irwin Clark, Columbia University College of Physicians and Surgeons
    AGENTS EFFECTING THE REMOVAL OF SKELETAL RADIOCALCIUM AND RADIOPHOSPHORUS IN INTACT AND PARATHYROIDECTOMIZED RATS
11. D. H. Copp
    PARATHYROIDS AND CALCIUM HOMEOSTASIS

2:00 P.M.

PARATHYROIDS AND CITRIC ACID

Chairman: *A. B. Hastings, Scripps Clinic and Research Foundation*

12. Smith Freeman, Northwestern University School of Medicine
    CITRIC ACID AND CALCIUM METABOLISM
13. Alexander Kenny, Harvard University School of Medicine
    CITRIC ACID PRODUCTION BY BONE
14. W. F. Neuman, University of Rochester
    POSSIBLE FUNDAMENTAL ACTION OF PARATHYROID HORMONE IN BONE

7:00 P.M.

BANQUET—RICE INSTITUTE MEMORIAL CENTER

Moderator: *Arthur Grollman, Southwestern Medical College*

Speaker: *Pieter J. Gaillard, State University, Leiden*
"PARATHYROIDS AND BONE IN TISSUE CULTURE"

SATURDAY, FEBRUARY 6, 1960

9:00 A.M.

PARATHYROID RESEARCH AT RICE INSTITUTE

Chairman: *Roy V. Talmage, Rice Institute*

16. G. D. Buchanan, University of Tennessee Medical Units
PARATHYROID INFLUENCES ON RENAL EXCRETION OF CALCIUM
17. Richard Davis, Rice Institute and Rockefeller Institute
MICROSCOPIC AND ULTRAMICROSCOPIC ANATOMY OF THE PARATHYROIDS
18. R. J. Toft, Rice Institute
THE PARATHYROIDS AND OSTEOCLAST FORMATION
19. Roy V. Talmage, Rice Institute
THE PROBLEM OF CONTROL OF PARATHYROID SECRETION

2:00 P.M.

CLINICAL PROBLEMS OF PARATHYROID MALFUNCTION

Chairman: *Frank L. Engel, Duke University School of Medicine*
Panel Member: *E. C. Reifenstein, Jr., Squibb Inst. for Med. Res.*

20. B. M. Black, Mayo Clinic
PATHOLOGY AND SURGERY OF THE PARATHYROID GLANDS
21. Ed Yendt, University of Toronto Department of Medicine
RELATIONSHIP OF URINARY PHOSPHATE CHANGES TO PARATHYROID ACTIVITY
22. J. E. Howard, Johns Hopkins Hospital
CLINICAL MANIFESTATIONS OF HYPERPARATHYROIDISM
23. *Panel Discussion:* Engel, Black, Yendt, Howard, Riefenstein

SECOND VENEZUELAN CONGRESS ON OBSTETRICS AND GYNECOLOGY

The Second Venezuelan Congress on Obstetrics and Gynecology will be held in Caracas, Venezuela, *February 19–24, 1960*. It is being organized to celebrate the 20th Anniversary of the Obstetrical and Gynecological Society of Venezuela. In Obstetrics, the main subjects of discussion will be Anemia in Obstetrics, Induction of Labor, Perinatal Mortality, and Evaluation of Thierry's Spatules. In Gynecology, the main subjects will be Pathology of the Tubes, Early Diagnosis of Cancer of the Cervix, Laparoscopy, and Gynecography. There will be several round-table discussions, and conferences by foreign and Venezuelan honor guests. For information, write to the Secretary, Dr. H. Marcano Guzmán, Apartado 7332 (San Martín), Caracas, Venezuela.

THE AMERICAN COLLEGE OF PHYSICIANS

The American College of Physicians will give a Postgraduate Course in "Recent Advances in Metabolic Diseases," *February 8–12, 1960*, at The Mount Sinai Hospital, New York, N. Y.—Dr. Alexander B. Gutman, Director. For information, apply to The American College of Physicians, 4200 Pine Street, Philadelphia 4, Pa.